Primary Structure and Expression Studies of the Dihydrofolate Reductase Gene (*DFR1*) of *Saccharomyces cerevisiae*

by

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ABSTRACT

The nucleotide sequence of a genomic DNA fragment thought previously to contain the dihydrofolate reductase gene (DFR1) of Saccharomyces cerevisiae by genetic criteria was determined. This DNA fragment of 1784 basepairs contains a large open reading frame from position 800 to 1432, which encodes a enzyme with a predicted molecular weight of 24,229.8 Daltons. Analysis of the amino acid sequence of this protein revealed that the yeast polypeptide contained 211 amino acids, compared to the 186 residues commonly found in the polypeptides of other eukaryotes. The difference in size of the gene product can be attributed mainly to an insert in the yeast gene. Within this region, several consensus sequences required for processing of yeast nuclear and class II mitochondrial introns were identified, but appear not sufficient for the RNA splicing. The primary structure of the yeast DHFR protein has considerable sequence homology with analogous polypeptides from other organisms, especially in the consensus residues involved in cofactor and/or inhibitor binding. Analysis of the nucleotide sequence also revealed the presence of a number of canonical sequences identified in yeast as having some function in the regulation of gene expression. These include UAS elements (TGACTC) required for the amino acid general control response, and "TATA" boxes as well as several consensus sequences thought to be required for transcriptional termination and polyadenylation. Analysis of the codon usage of the yeast DFR1 coding region revealed a codon bias index of 0.0083. this valve very close to zero suggestes

that the gene is expressed at a relatively low level under normal physiological conditions.

The information concerning the organization of the DFR1 were used to construct a variety of fusions of its 5' regulatory region with the coding region of the lacZ gene of E. coli. Some of such fused genes encoded a fusion product that expressed in E.coli and/or in yeast under the control of the 5' regulatory elements of the DFR1. Further studies with these fusion constructions revealed that the beta-galactosidase activity encoded on multicopy plasmids was stimulated transiently by prior exposure of yeast host cells to UV light. This suggests that the yeast DFR1 gene is induced by UV light and may imply a novel function of DHFR protein in the cellular responses to DNA damage.

Another novel feature of yeast DHFR was revealed during preliminary studies of a diploid strain containing a heterozygous DFR1 null allele. The strain was constructed by insertion of a URA3 gene within the coding region of DFR1. Sporulation of this diploid revealed that meiotic products segregated 2:0 for uracil prototrophy when spore clones were germinated on medium supplemented with 5-formyltetrahydrofolate (folinic acid). This finding suggests that, in addition to its catalytic activity, the DFR1 gene product may play some role in the anabolism of folinic acid. Alternatively, this result may indicate that Ura+ haploid segregants were inviable and suggest that the enzyme has an essential cellular function in this species.

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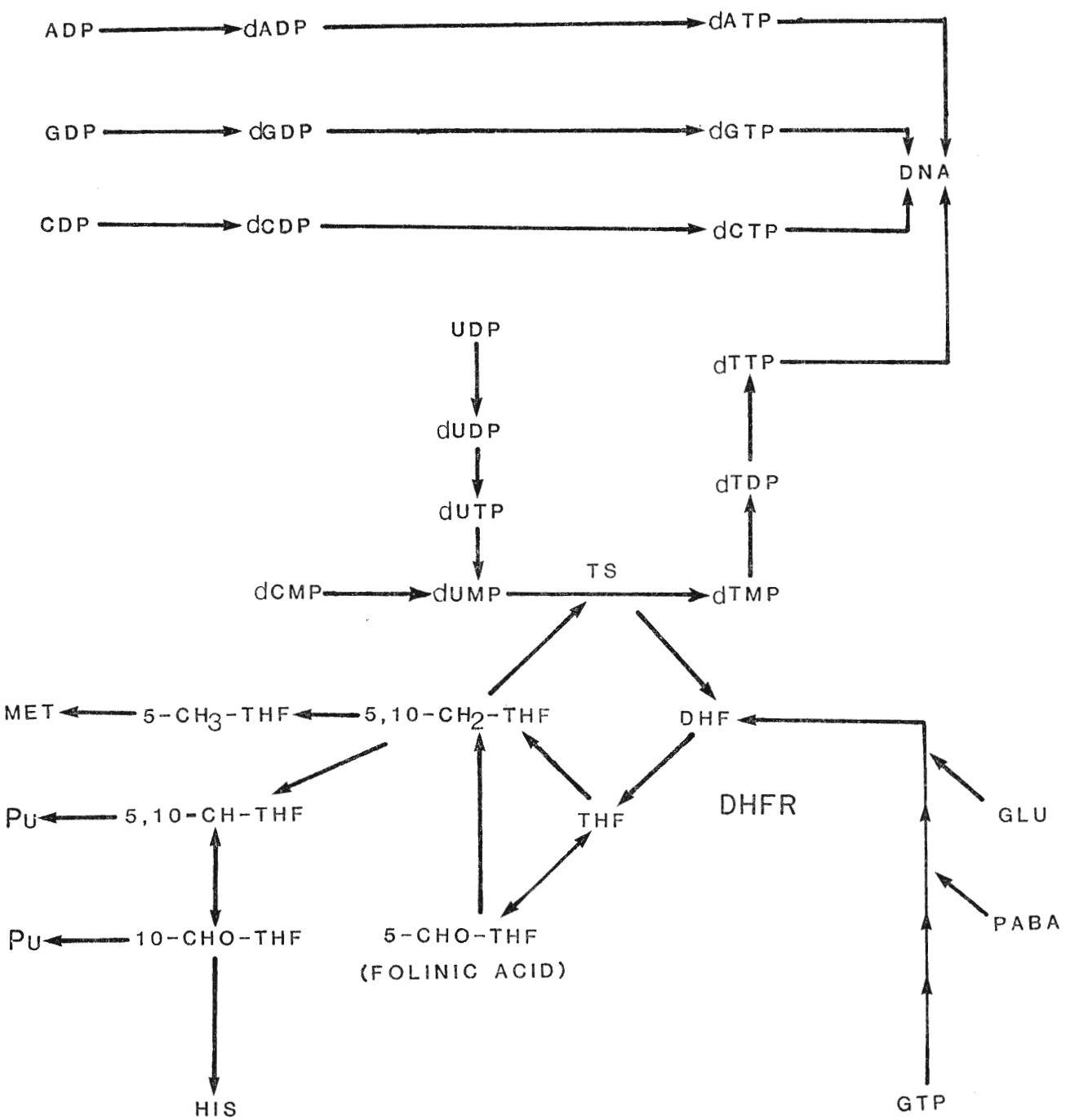
INTRODUCTION

Dihydrofolate reductase (DHFR; EC 1.5.1.3) has an essential role in cellular metabolism and growth. A major function of this enzyme is to maintain the intracellular pools of reduced folate cofactors by reducing dihydrofolate (DHF) to tetrahydrofolate (THF), utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as coenzyme. Intracellular dihydrofolate may be formed either through de novo biosynthesis or by the action of thymidylate synthase (TS). The reduced product (THF), in turn, serves as a source of metabolically derived THF coenzymes such as 5,10-methylene-THF, 5 or 10-formyl-THF and 5-methyl-THF. Each of these THF cofactors is formed from a particular carbon source and THF, or derived directly from a metabolic derivative of THF. These THF coenzymes are required for a number of one-carbon unit transfer reactions in the biosynthesis of a variety of essential cellular metabolites including amino acids, purine and the pyrimidine nucleotide thymidine 5'-monophosphate (see Fig.1; Blakley, 1969; Chabner, 1982). In addition, a reduced folate is the donor in the formylation of met-tRNA-FMET required in the initiation of protein synthesis in prokaryotes and in the mitochondria of eukaryotes. After reaction of these THF cofactors with the enzymatic substrate, a new product and unsubstituted THF are formed. Thus, in most interconversion reactions, THF can be considered as a carrier of one-carbon units, shuttling back and forth between the 'loaded' and 'empty' states. In contrast to the reaction mechanisms of these folate dependent enzymes, in which the folate cofactor remains at the fully reduced level,

Figure 1 The role of DHFR in cellular metabolism


ADP
dADP
dATP
GDP
dGDP
dGTP
DNA
CDP
dCDP
dCTP
UDP
dTTP
dUDP
dTDP
dUTP
dCMP
dUMP
TS
dTMP
MET
5-CH3-THF
5,10-CH2-THF
DHF
Pu
5,10-CH-THF
THF
DHFR
GLU
PABA
Pu
10-CHO-THF
5-CHO-THF
(FOLINIC ACID)
HIS
GTP

thymidylate synthase catalyzes the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) by reaction with the methyl donor 5,10-methylene-THF. Concomitantly, THF is oxidized to DHF. Thus, the action of DHFR is also required for the regeneration of THF from the DHF formed in this manner (Fig.1). This requirement is essential for all cells, but is especially critical in yeast, as, in common with most fungi, they lack thymidine kinase activity and thus possess no salvage pathway for the anabolism of thymine or thymidine (Grivell and Jackson, 1968). As a consequence, in yeast, all intracellular dTMP is derived ultimately from dUMP. In contrast, mammalian cells can synthesize dTMP either by de novo synthesis from dUMP or from dietary thymidine via the salvage pathway. As replicating cells have an absolute requirement for dTMP for DNA synthesis, DHFR must reduce DHF as rapidly as it is formed by TS to resupply THF for dTMP synthesis and to avoid depletion of intracellular THF derivatives required for other biosynthetic reactions.

Due to this key role in cellular metabolism and growth, DHFR has long been of significant pharmacological interest as the target for a number of chemotherapeutic agents. These include the antineoplastic methotrexate (MTX), the antibacterial trimethoprim (Tm) and the antimalarial pyrimethamine, all of which are potent inhibitors of the enzyme (Roth, 1983; Rollo, 1983; Calabresi and Parks, 1985). Competitive binding of these inhibitors to DHFR in vivo results in depletion of intracellular THF pools, a consequence of which is a block in de novo synthesis of purine nucleotides and dTMP, thereby

inhibiting DNA synthesis. Ultimately, cells cease dividing and lose viability (Blakley, 1969). Thus, these drugs have been employed extensively in chemotherapeutic treatments of neoplastic disease, bacterial infection and a variety of other clinical disorders (Bertino and Johns, 1972; Roth and cheng, 1982; Hitchings, 1983).

Because of the pharmacological importance of the enzyme, much effort has been directed towards an understanding of the nature of inhibition of DHFR by various chemicals. Thus, it has been found that bacterial and eukarotic DHFRs show a wide range of sensitivity to drugs such as Tm (Burchall, 1974; Baccanari et al., 1982). In addition, numerous studies have shown that bacteria often develop resistance to Tm by increased production of a DHFR isozyme which is not the predominant species in the parental strain (Hutchison, 1971). Collectively, these observations have led to an impetus to understand the underlying molecular basis for the differential effects of drug inhibition of various DHFR enzymes. It is the hope of investigators taking this approach that such studies may provide some insight into the structure of the DHFR protein and its molecular pharmacology in a variety of cell types and lead to the design of more efficacious clinical agents with increased selectivity.

The progress of such studies has been facilitated by the fact that DHFRs are relatively small proteins with molecular weights ranging from 15,000 to 30,000 Daltons depending on the source (Blakley, 1969). In addition, resistance to MTX or Tm in both bacterial and animal cells is often accompanied by a marked

increase in the production of DHFR. Isolation of drug-resistant mutants, therefore, has often provided a convenient source of the large quantities of the protein required for detailed studies of enzyme mechanics. In addition, the availability of the highly purified enzyme has made it possible to obtain crystals of the protein for X-ray crystallographic studies. Thus, the crystal structure of binary complexes of bacterial and animal DHFRs with different inhibitors or with the cofactor NADPH (Filman et al., 1982; Baker et al., 1981 and 1983; Matthews et al., 1977, 1979 and 1985a), and the ternary complexes of bacterial and animal DHFRs with various inhibitors and NADPH (Mattlews et al., 1978 and 1985b; Bolin et al., 1982; Voltz et al., 1982; Champness et al., 1986) have been determined. These X-ray crystallographic studies have suggested that the differential sensitivities of the animal and bacterial enzymes to Tm and MTX can be accounted for by slight differences in the portions of the enzyme structure which interact directly with the inhibitors.

Because of the relative ease with which DHFR protein has been purified from mutant cells which overproduce DHFR in a variety of organisms, it is not surprising that during the last decade or so, the complete primary structure of a number of bacterial and animal DHFRs has been determined (see Appendix I). Combined with the X-ray crystallographical data, comparative studies of the amino acid sequences of DHFRs, from various sources, have shown that among the amino acid residues involved in inhibitor and NADPH binding, a small number of them are shared by both bacterial and animal enzymes (Volz et al., 1982). The

important role of some of these consensus residues in the catalytic activity of the enzyme and its inhibition by a variety of chemical agents has been confirmed by analysis of mutant enzymes and chemical modification experiments, as well as by directed mutagenesis studies (Freisheim et al.,1977; Baccanari et al.,1981; Simonsen and Levinson, 1983; Villafranca et al., 1983; Chen et al., 1985; Howell et al., 1986). Furthermore, bacterial and animal DHFR complexes with Tm and MTX or substrate have also been studied extensively by nuclear magnetic resonance (Birdsall et al. 1977 and 1983; Wyeth et al., 1980; Cocoo et al., 1981). Data from these studies have contributed greatly to the elucidation of the detailed molecular structure of various DHFR enzymes as well as to an increased understanding of the mechanics of enzyme catalysis and inhibition. Consequently, DHFR has rapidly become, without question, the best-understood enzyme involved in folate intermediary metabolism, in terms of its physical biochemistry.

In addition to numerous physical and biochemical studies of the DHFR enzyme, attention to the control of the expression of the DHFR gene was stimulated by the finding that the use of MTX and Tm during chemotherapy was often compromised by the emergence of cells resistant to the inhibitory effects of these drugs. Research into DHFR gene expression was predicated upon the belief that studies of the detailed molecular mechanism of drug resistance might lead to improved strategies for the development of new therapies that selectively kill resistant cells. In numerous studies, the development of antifolate drug resistance

in variety of cell populations was shown to result from any one of at least four mechanisms acting separately or in concert.

First, mutations that impaired drug uptake resulted in a resistant phenotype (Frei et al., 1984). Second, resistance in bacterial cells was shown to be due to the presence of plasmids (R-factors) that coded for drug-insensitive DHFRs. It was concluded that the presence of such a plasmid provided an alternative nonsusceptible enzyme that permitted bypass of the metabolic block imposed by the inhibited target DHFR of the host bacterium. To date, four distinct types of DHFR encoded by R-factors have been detected in a variety of clinical isolates of bacteria.

Type I DHFR was only inhibited by Tm and MTX at levels several-thousand-fold greater than those which inhibited the enzyme encoded by the bacterial host. The active form of the enzyme was a dimer with native molecular weight of 35,000 Daltons, which was approximately twice that of the chromosomally encoded DHFR (Patlishall et al., 1977; Fling and Elwell, 1980). The amino acid primary structure deduced from the nucleotide sequence of the type I DHFR gene, specified by plasmid R 483, showed that the enzyme was most homologous to wild-type bacterial DHFRs in the regions thought to be involved in substrate binding, but showed relatively little homology in the areas lying outside the active site (Simonsen et al., 1983). Type II DHFR was almost completely insensitive to the effect of Tm and MTX and was a tetramer with a native molecular weight of 35,000 Daltons (Pattishall et al., 1977; Smith et al., 1979). The amino acid

primary structure (Stone and Smith, 1979) and nucleotide sequence (Swift et al., 1981) analysis of type II DHFR, specified by the plasmids R67 and R388, respectively, revealed no homology with any other known DHFRs from bacterial or animal sources. Both type I and type II plasmid-encoded DHFRs showed little difference from the bacterial enzyme in their binding affinities to the substrate (DHF) or to the cofactor (NADPH) (Pattishall et al., 1977). In contrast to these plasmid-encoded DHFRs, type III enzyme , specified by plasmid pH21, had a high affinity for DHF and confered only moderate resistance to Tm upon the host. However, it showed extreme sensitivity to MTX inhibition (Fling et al., 1982; Joyner et al., 1984). Recently, a type IV DHFR, encoded by plasmid pUK1123, has been characterized (Young and Amyes, 1986). Similar to the type III DHFR, the type IV enzyme confered only a moderate level of Tm resistance upon its host, but showed a marked size difference from all other plasmid DHFRs. It had a relatively high molecular weight of 46,700 Daltons and its synthesis was induced in a dose-dependent manner by increasing concentrations of Tm.

A third mechanism of drug resistance in both bacterial and animal cells was observed to be due to the expression of a novel DHFR, exhibiting a decreased affinity for the inhibitor, or a form of DHFR with increased substrate specificity (Flintoff and Essani, 1980; Goldie et al., 1981; Haber et al., 1981). Analysis of the mutant DHFRs found in a Tm resistant strain of E. coli (Baccanari et al., 1981) and in a MTX-resistant line of mouse 3T6 cells (Simonsen and Levinson, 1983) have shown that the altered

DHFR found in each organism differs from the wild-type enzyme only at a single amino acid substitution, in the region of the polypeptide thought to be essential for inhibitor binding. Recently, an activity-increased form of DHFR was also found in a MTX-resistant line of human promyelocytic-leukaemia (HL60) cells (Dedhar et al., 1985).

The final variant exhibiting the drug-resistant phenotype, observed in these studies, resulted from overproduction of DHFR, caused either by an increase in the expression of the DHFR gene at the level of transcription (Sirotnak and McCuen, 1973; Sheldon and Brenner, 1976) or by gene amplification (Alt et al., 1978). Studies in bacteria revealed that enzyme overproduction was often associated with mutations near the structural gene (McCuen and Sirotnak, 1974; Sheldon, 1977; Smith and Calvo, 1982) which was shown to result in a corresponding increase in the rate of DHFR gene transcription (Sirotnak and McCuen, 1973; Smith and Calvo, 1979). Analysis of the nucleotide sequence alterations that were responsible for Tm resistance in these E. Coli mutant strains indicated that changes had occured in the promoter region of the DHFR gene which had resulted in an increased homology of this region to the E. coli promoter consensus sequence. It was thought that these alterations gave rise to more efficient RNA polymerase binding at this mutant promoter and thus to an increase in the level of DHFR gene expression (Smith and Calvo, 1982).

In contrast to the results observed in bacteria, over-production of DHFR in mammalian cells challenged with MTX was

often associated with a corresponding increase in DHFR-specific mRNA as a consequence of gene amplification, (Alt et al., 1978; Schimke, 1984). In some cells, the amplified genes were lost upon continued growth in the absence of MTX, whereas, in other cell lines, the amplified genes were stable, when the cells were cultured under non-selective conditions. Subsequently it was shown that the stably amplified genes were localized to expanded loci called "homogeneously staining regions" in one or more chromosomes (Nunberg et al., 1978; Masters et al., 1982). In unstable cells, no change was seen in the chromosomes carrying the DHFR gene and the amplified DHFR DNA sequences were associated with large numbers of small extrachromosomal elements called "double-minute chromosomes" (Kaufman et al., 1979; Masters et al., 1982). Presently, the role of gene amplification in the development of this mode of MTX resistance and the molecular mechanism of gene amplification remains unclear. Several models have been proposed in an attempt to explain these findings, including unequal sister chromatid exchange or disproportionate DNA replication, although conclusive evidence for either model is lacking (Schimke, 1984).

The results of studies, in last a few years, of the physical structure of the various DHFR genes has greatly facilitated studies of control of DHFR expression and the mechanism of gene amplification. Recombinant DNA technology, combined with other modern molecular approaches, has made it possible to study DHFR gene regulation in much more detail. Thus, during the past few years, several bacterial DHFR genes have been cloned and

sequenced (see Appendix II). In addition, several eukaryotic DHFR genes have also been isolated on chimeric plasmids. These include genes encoding enzymes from the mouse (Chang et al., 1978; Nunberg et al., 1980; Crouse et al., 1982), human (Chen et al., 1984; Yang et al., 1984) and Chinese hamster cells (Carothers et al., 1983; Metera et al., 1984) as well as from the baker's yeast S. cerevisiae (Nath and Baptist, 1984; Barclay, personal communication). Moreover, cloning and sequencing of a gene coding for a bifunctional DHFR-TS protein from L. major was recently reported (Coderre et al.,1983; Beverley et al., 1986).

The mouse, human and Chinese hamster DHFR genes have been extensively characterized. All three genes are relatively large, comprising 34 kb of genomic DNA for the mouse gene, 30 kb for the human chromosomal DNA and 26kb for Chinese hamster DHFR locus. In general terms, all three genes have a similar overall structure, i.e. they contain the same number of exons (six) which comprise a 561 bp nucleotide coding sequence for mouse DHFR and 564 bp for both the human and Chinese hamster genes. All three genes contain five introns, at equivalent positions within the coding sequence. The different overall sizes of the three genes is attributable mainly to the different lengths of their introns (Yang et al., 1984).

Analysis of the regulation of expression of these mammalian DHFR genes has revealed an previously unsuspected complexity. For example, it has been shown that the mouse DHFR gene is expressed as multiple mRNA species, consisting of four major and two minor transcripts (Setzer et al., 1980; Farnham and Schimke, 1986a).

Several of these mRNAs result from the use of different polyadenylation sites, yielding mRNA molecules that differ in the length of their 3' untranslated regions (Setzer et al., 1982; Masters et al., 1983). There is also apparent heterogeneity in the initiation site for mouse DHFR gene transcription (McGrogen et al., 1985; Sazer and Schimke, 1986). In human cells, three main species and at least six minor species of DHFR mRNA have been identified (Morandi et al.,1982; Masters and Addardi, 1985). The three major species reflect the utilization of a single transcription initiation site at the 5' end of the gene but three different polyadenylation sites in the 3' downstream region. The minor species can be accounted for as transcripts originating from sites for transcriptional initiation several hundred nucleotides upstream of the main mRNA start site (Masters et al., 1983; Masters and Attardi, 1985). Although the Chinese hamster transcription unit has not yet been as extensively characterized, multiple transcripts have been observed. In common with the previous studies, these seem to be related to the utilization of different transcription initiation sites and polyadenylation signals (Lewis et al., 1982; Carothers et al., 1983; Mitchell et al., 1986).

In addition to the apparent complexity of DHFR transcription initiation, functional analysis of the DNA sequence of the mammalian DHFR genes has revealed several other distinctive features of their promoter regions. Firstly, unlike most higher eukaryotic genes studied to date, mammalian DHFR genes lacked typical "CAAT" and "TATA" boxes upstream from the transcriptional

initiation site. Alternatively, novel 29 bp conserved sequences were found at a relatively fixed location 45 to 49 bp upstream of each major transcription start site of the various genes. One such conserved sequence was found in the human DHFR gene (Chen et al.,1984; Yang et al., 1984); two repeats were located in the Chinese hamster gene (Mitchell et al., 1986) and four similar regions were seen in the mouse gene (Crouse et al., 1982). Each of these conserved sequences contained an identical "GC" box thought to be the binding site for the eukaryotic RNA polymerase II transcription factor Sp1 (Dynan and Tjian, 1985). Another novel feature of both the mouse and hamster genes was the finding that the DHFR promoter functions bidirectionally, with transcription occuring divergently on opposite DNA strands (Farnham et al., 1985; Crouse et al., 1985; Mitchellet et al., 1986). The significance of the bidirectionality of transcription and the identification of the divergent gene product remain to be elucidated.

In addition to the interesting regulatory elements that have been identified in their promoter regions, examination of the 3′ end of various DHFR mRNA species and the DNA sequence surrounding the polyadenylation sites have indicated other novel features of the transcription unit of DHFR genes (Setzer et al., 1982; Masters et al., 1983). Thus, it has been reported that DHFR mRNA transcription was terminated at multiple sites and that some of the polyadenylation signals were located several hundred to several thousand nucleotides downstream from the translation terminator. Moreover, the canonical sequence "AAUAAA", thought to

be the polyadenylation signal for most eukaryotic mRNAs is lacking in many of these DHFR mRNA species. This suggests that an alternative signal is required for the polyadenylation of the mammalian DHFR mRNAs. A possible explanation for these unusual features of the structure of various DHFR genes is that these sequences might account for the observed complexity of the regulation of their expression.

DHFR genes have been classified as "housekeeping" genes that are normally constitutively expressed at a relatively low level. Although expression of DHFR genes at a basal level seems necessary for the intermediary metabolism of one carbon transfer reactions, many studies have shown that intracellular DHFR levels are responsive to a variety of modulators (Cowan et al., 1986), such as viral infection (Kellems et al., 1979; Yoder et al., 1983; Yoder and Berget, 1985), treatment with folate antagonists (Chello et al., 1976; Domin et al., 1982) or serum (Gudewicz el al., 1981; Santiago et al., 1984). In addition, it has been shown that, in common with a number of enzymes involved in DNA replication and deoxyribonucleotide synthesis, the activity of DHFR in mammalian cells is expressed periodically during the cell cycle. Maximal DHFR activity occurred during S phase (Hillcoat et al., 1973; Alt et al., 1976; Johnson et al., 1978). Further studies showed that the increase in DHFR level was due to an increased rate of <u>de novo</u> DHFR synthesis rather than to stabilization of the enzyme (Alt et al., 1976; Mariani et al., 1981). Also, it is generally agreed among investigators in the field that the increased rate of DHFR synthesis in S phase

reflects an increase in the rate of DHFR messenger production rather than from an increase in the half-life of DHFR mRNA (Hendrickson et al., 1980; Leys and Kellems, 1981; Mullner et al., 1983).

However, the level at which DHFR mRNA producton is regulated has been in dispute for a number of years. The main point of contention is whether the rate of production of DHFR mRNA is controlled by regulation of the rate of DHFR transcription, or by modulation of transcript processing and/or export of the mature DHFR mRNA from the nucleus to the cytoplasm. Both conclusions have some support from the experimental data obtained to date.

In studies of the DHFR gene in stationary phase sarcoma 180 cells following replating at lower cell density, Leys and Kellems (1981) showed that growth stimulation resulted in a threefold increase in the rate of mature DHFR mRNA production. In addition, these authors showed that this increase was not accompanied by a corresponding change in the rate of transcription of the DHFR gene. Subsequent studies (Leys et al., 1984) revealed that, in stationary cells, most DHFR transcripts were rapidly degraded in the nucleus, but there was no significant difference in the rate of processing and transport of stable DHFR messenger. As a consequence of these findings, these authors concluded that changes in the stablity of DHFR mRNA in the nucleus ultmately controlled the amount of DHFR mRNA available for transcription in the cytoplasm. Additional support for this view resulted from similar observations in studies of amino acid-starved 3T6 cells (Collins et al., 1983). Moreover, studies of the expression of a

chimeric SV40-mouse DHFR cDNA gene introduced into DHFR deficient Chinese hamster cells demonstrated that the increased rate of production of DHFR mRNA in growing cells was not associated with changes in the level of transcriptional activity (Kaufman and Sharp, 1983). Furthermore, these studies showed that the 3' noncoding region of the DHFR mRNA appeared to be involved in the control of the conversion of DHFR hnRNA into mature mRNA, in stationary phase cells. Thus, these experiments suggested that DHFR mRNA production was controlled at the posttranscriptional level during the cell cycle.

In contrast to these observations were other reports which documented a good correlation between the rate of DHFR gene transcription and the rate of cytoplasmic DHFR mRNA production in serum-stimulated 3T6 cells (Santiago et al., 1984). A similar conclusion was also reached during analysis of DHFR gene transcription in mouse 3T6 cell populations synchronized in different phases of the cell cycle (Farnham and Schimke, 1985). More recent studies have demonstrated that intracellular levels of all of the mouse DHFR transcripts were maintained throughout the cell cycle in a similar manner. Thus, DHFR messenger levels increased at the G1/S boundary without significantly changing their ratios relative to one another, in both the nucleus and in the cytoplasm (Farnham and Sckimke, 1986a). Furthermore, transfection experiments with various DHFR minigenes have revealed that the DNA sequence upstream from the transcription site was required for this cell-cycle regulation (Farnham and Schimke, 1986a).

The observation that there was no differential utilization of transcriptional initiation sites of the various DHFR mRNAs in different phases of the cell cycle has led to the suggestion that cell-cycle regulation of the DHFR gene may be mediated by a transient increase in the general efficiency of transcription at the DHFR promoter. Thus, the periodicity of DHFR mRNA production may reflect regulation by a transcriptional factor whose activity changes as cells progress through the cell cycle (Farnham and Schimke, 1985). Consonant with this view, Farnham and Schimke (1986a) have since demonstrated the presence of a cell-cycle-specific factor which stimulated transcription at the DHFR gene in an _in vitro_ transcription system. Additional experimental support for the model has recently obtained by Dynan et al. (1986) who also have shown that the eukaryotic transcription factor Sp1 can specifically stimulate transcription from the DHFR promoter. However, the establishment of a definitive role for the Sp1 factor in the cell cycle regulation of DHFR gene will still require further investigation. Specific experiments are required to determine whether the activity of Sp1 also exhibits a cell cycle pattern and to isolate and characterize other factor(s) which may interact with Sp1 during the cell cycle. Whatever the detailed molecular mechanism might be, the results described above seem to indicate that the rate of production of DHFR mRNA is controlled by at least two different mechanisms which respond to the physiological state of the cell.

An additional feature of interest, with respect to DHFR gene expression, is the possible role of an autoregulatory mechanism

in the control of DHFR synthesis (Sirotnak, 1970; Calvo and Fink, 1971; Sirtnake and McCuen, 1973; Gronenborn and Davis, 1981; Bastow et al., 1984). Experimental evidence which supports the notion of autogenous regulation came from the finding that treatment with subsaturating doses of MTX was shown to induce intracellular DHFR activity by increasing DHFR synthesis rather than by gene amplification. Thus, it has been proposed that MTX or a metabolic derivative could prevent DHFR from exerting its autoregulatory activity by altering the enzyme conformation or, alternatively, by competing with a regulatory molecule (Bastow et al., 1984). In this way, intracellular DHFR activity could be controlled either at the transcriptional and/or post-transcriptional level. Thus, feedback control could occur through direct interaction of DHFR with its mRNA or with a DHFR-specific DNA sequence. There is no experimental evidence that supports the idea of an interaction of DHFR with its own message, but there is some data which suggests that the L. casei DHFR protein has DNA binding acitivity, with a greater affinity for a DNA sequence 5′ to the DHFR structural gene (Gronenbon and Davis, 1981).

Although we do not have a complete picture of the various elements which modulate DHFR gene expression, results to date indicate that the control of DHFR expression is apparently quite complex. In spite of a general lack of knowledge about detailed molecular mechanisms, activity studies have begun to provide some important insights into the control of DHFR expression. It is clear that advances in our understanding of DHFR regulation will

not only contribute to our basic knowledge about eukaryotic gene expression in general, but, because of the importance of DHFR as a target of many chemotherapeutic drugs, it is likely that such knowledge will also be of some clinical utility.

Although DHFR proteins and genes from various organisms have been studied in some depth, relatively little is known about the enzyme or the gene which encodes it in the baker's yeast Saccharomyces cerevisiae, which, as an experimental system, has great technical advantages. In early experiments, by studying the enzymatic defects in folate-dependent yeast auxotrophs, Zelikson and Luzzati (1977) suggested that there are two parallel sets of enzymes involved in folate coenzyme-mediated one-carbon transfer. According to these authors, one set of folate enzymes is located in the mitochondria and the other set is found in the cytoplasm. Among the THF interconversion enzymes studied was DHFR. It is clear that such dual subcellular distributions of the enzyme activity requires further experimental confirmation. However, if supported by additional experimentation, these results raise the interesting possibility that more than one gene may encode DHFR activity in yeast or alternatively that a single genetic locus may encode two DHFR activities. Although no DHFR- yeast mutant has been isolated, the phenotype of which might shed some light on this question, the wild type allele of the gene (DFR1) has been isolated on a chimeric plasmid. The selection scheme was based on the differential sensitivties to Tm of the E.coli and yeast DHFRs (Nath and Baptist, 1984; Barclay, personal communication). Preliminary studies using the

cloned gene (Barclay and Nagel, manuscript in preparation) have shown that no effect on host Tm sensitivity can be observed when a plasmid harboring the DFR1 gene is introduced into rad6, rad18 (Game et al., 1975) or saline-treated RAD wild-type yeast strains (Mayer and Goin, 1984). This was a somewhat surprising result as there was a significant increase in resistance to MTX, another DHFR inhibitor, in these strains grown under similar conditions. These observations mentioned above were virtually all that was known about yeast DHFR expression at the beginning of this study.

It was my hope that the availability of many relatively sophisticated methods in classical genetics which have been developed in yeast over many years, combined with the more novel and very powerful techniques in modern molecular biology, would afford many technical advantages to studies of DHFR expression in yeast. Two very useful techniques which have been developed in recent years are gene fusion and gene disruption, or gene transplacement.

Gene fusions were originally developed in prokaryotic systems and since that time have been used with considerable success in yeast. E.coli lacZ (encoding beta-galactosidase) fusions have been most commonly used because of the many advantages afforded in the construction of required fusions and in the biochemical assay of their products. For example, assay systems which are not only convenient but also extremely sensitive have been developed for qualitative and quantitative measurements of beta-galactosidase activity (Miller, 1972). In

addition, it has been demonstrated that as many as 27 amino acids at the amino terminus of the beta-galactosidase protein may be replaced with other peptide sequences of varying length without substantially affecting the activity of the enzyme (Brickman et al., 1979). Thus, the coding region of the lacZ gene can easily be fused to a DNA sequence containing the translational start codon and a promoter of interest as well as attendant 5' regulatory elements, so that expression of the lacZ gene is now under the control of the genetic regulator under study. Factors affecting the level of expression of a particular gene of interest can be monitored by observing changes in beta-galactosidase activity under various experimental conditions. Thus, it has now become commonplace to engineer hybrid genes between yeast genes and prokaryotic genes (such as the lacZ gene) and to insert these fusions into shuttle plasmids that can be moved back and forth relatively easily between E.coli and yeast. Furthermore, studies from several laboratories have demonstrated that the lacZ gene fusions are readily expressed in yeast cells, which themselves have no inherent beta-galactosidase activity (Rose et al., 1981; Guarente and Ptashne, 1981; Guarente, 1983). Because of their versatility and resolving power, lacZ gene fusions have been employed in a wide range of experiments in yeast, including studies of protein localization, protein transport and gene expression. Studies of gene fusion expression have been particularly valuable for the detection of regulatory elements of genes and the molecular signals which regulate gene expression, which may have been

difficult to study by other methods.

In addition to gene fusion studies in yeast, other techniques utilizing cloned genes have been developed, which greatly facilitate the studies of genetic regulation in this simple eukaryote. One of the more powerful techniques is the use of integrating plasmids to mutagenize yeast chromosomal loci by gene disruption or transplacement (Scherer and Davis, 1979; Rothstein, 1983; Struhl, 1983; Boeke, 1984; Rudolph et al., 1985). The basic feature of this approach is that any cloned DNA fragment, after appropriate manipulation in vitro, can be reintroduced into yeast cells and integrated into the original locus through homologous recombination. There are many useful applications of these gene disruptions or gene transplacements. For example, once a cloned yeast DNA fragment has been appropriately engineered, it can be used to alter or delete completely a specific chromosomal region, thereby introducing a mutant allele into a particular genetic background. Thus, it would be possible to determine whether a gene has an essential function in yeast, as such a disruption in the heterozygous state would segregate 2:0 for the lethal phenotype. Such studies employing the cloned DFR1 yeast gene would greatly improve our understanding of the role of DHFR in cellular metabolism.

A long term objective of studies in our laboratory has been to understand the genetics and biochemistry of pyrimidine nucleotide and folate metabolism in yeast. Recently, it has been shown that thymidylate stress has considerable genetic and

biochemical effects (Barclay et al.; 1982, Kunz, 1982; Haynes, 1985). In yeast, depletion of thymidylate pools causes "thymineless death", and is highly recombinagenic for nuclear genes and is mutagenic for the mitochrondrial genome (Barclay and Little, 1978). Excess thymidylate is highly mutagenic for nuclear genes (Barclay and Little, 1981). Several folate antagonists such as MTX, sulfanilamide (SULF) and aminopterin cause a depletion of the intracellular THF pools, which ultimately results in "thymineless death" as well. One of the difficulties in our attempt to understand the nature of the genetic and biochemical effects of thymidylate stress is a paucity of knowledge about the regulation of genes which encode the enzymes involved in thymidylate and folate metabolism. A very important enzyme in this pathway is DHFR. Thus, knowledge of the expression and regulatory mechanism of DHFR synthesis is essential for our increased understanding of the regulation of folate and pyrimidine nucleotide metabolism in this organism.

The availability of the cloned DFR1 gene on a chimeric plasmid enabled me to utilize the technical advantages of the yeast system in studying the regulation of this gene. As a first step towards the investigation of the regulation of DFR1 expression at the molecular level, it was necessary to characterize the physical structure of the gene. It was thought that identification of the coding region of the gene and its continuous regulatory elements would be useful in the construction of DFR1:lacZ gene fusions for use in further studies of yeast DHFR gene regulation. Moreover, this information

could also be used for the construction of a gene disruption vector to be used in the isolation of a DHFR-deficient yeast strain, which would be valuable for biochemical and genetic analysis of DHFR function. In spite of the fact that much effort has been made in an attempt to isolate such a DHFR-deficient mutant (Little, personal communication), to my knowledge, no such mutant has been isolated by conventional classical techniques.

Thus, my thesis research began with the following intentions:

(1) Determination of the primary nucleotide sequence of the yeast DFR1 gene and attendant regulatory elements.

(2) Construction of a gene fusion between the coding region of the E.coli lacZ gene and the regulatory region of the yeast DFR1 gene.

(3) Construction of a DHFR-deficient yeast mutant strain by gene disruption.

It was my hope that by a combination of these approaches, I could develop useful molecular tools for more detailed investigations of the regulation of DHFR gene expression in this simple eukaryote.

MATERIALS AND METHODS

I. Enzymes and Chemicals

The large fragment of E.coli DNA polymerase I (Klenow fragment), restriction endonucleases, T4 DNA ligase and Bal31 exonuclease were obtained from Bethesda Research Labs, Inc. Glusulase was purchased from E.L. du Pont de Nemoours & Co. (Inc.). Zymolyase was supplied by Seikagaku Kogyo Co.. RNase T1 and RNase A were obtained from International Biotechnologies, Inc. DNA sequencing primer, nucleotide reagents and BamH1 restriction endonuclease 5' phosphorylated linker were purchased from Pharmacia P-L Biochemicals, Inc. Radioactive compounds were supplied by New England Nuclear Corp. All culture media reagents were purchased from Difco, BDH Chemicals or Bethesda Research Labs, Inc. Other chemicals were obtained from Sigma Chemical Co. or Pharmacia P-L Biochemicals, Inc.

II. Bacterial and Yeast Strains

E.coli strains used in this study are listed in Table 1. The strain JF1754 provided by J. Freisen (University of Toronto) was used as a host for all plasmids. Strains MC1064 and MC1066 (Casadaban et al. 1983) were employed in gene fusion studies and were also used as hosts for the fusion plasmids. The bacterial strain JM103 was purchased from BRL Labs., Inc. and was used in M13 cloning and the generation of single strand DNA templates for DNA sequencing.

Table 1. Bacterial Strains used in this study

Strain	Genotype	Source/Ref.
JF1754	lac, gal, metB, leuB, hisB436, hsdR.	J.D. Freisen.
MC1064	Δ(lacIPOZYA)X74, galU, galK, strA , hsdR , Δ(ara,leu), trpC9830.	Casadaban et al., 1983.
MC1066	Δ(lacIPOZYA)X74, galU, galK, strA , hsdR , Δ(ara,leu), trpC9830, pryF74::Tn5(Km).	Casadaban et al., 1983.
JM103	Δ(lac proA,B)/F', proA,B, lacIgZ M15, thi, strA, sbcB15, supE44, traD36, hsdR4.	Bethesda Research Labs.

The haploid yeast strain M1/2B (Casadaban et al.,1983) was used in both gene fusion and gene disruption studies. Diploid strain TH4 was derived from a mating of strain LP2729-4B provided by L. Prakash (Rochester University) with strain DS1/38 from this laboratory (D.F. Steele) and was employed in gene disruption studies. Haploid strain DBY747 was obtained from R.H. Schiestl (Rochester University) and was used in gene disruption studies (see Table 2).

III. Growth, Selection and Assay Media

Preparation and composition of bacterial complete (LB) medium and minimal (M9) medium were as described by Miller (1972). When required in selective procedures, appropriate amino acids were added to a final concentration of 50ug per ml. Antibiotics were added to the media at 48°c after autoclaving or spread on the plates to the following final concentrations: ampicillin 50ug/ml, neomycin sulfate 200ug/ml, and trimethoprim 2ug/ml. The M9 minimal medium containing lactose as a carbon source was employed in the plate assay of the beta-galactosidase activities.

The yeast complex media (YPD) and synthetic minimal medium (SD) employed in this study were those described by Sherman et al.(1974). For preparation of the synthetic complete medium with various constituents, appropriate amino acids, adenine and uracil were added at the final concentrations suggested by Sherman et al.(1974). Unless otherwise stated, folinic acid was supplied at the standard level required for the yeast Fol mutant strains (ie.

Table 2. Yeast strains used in this study

Strain	Genotype	Source/Ref.
M1/2B	MATα, trp1-289, ura3-52.	Casadaban et al., 1983.
LP2729-4B	MATα, his3-Δ1, leu2-3, leu2-112, trp1-289, ura3-52, rad18-2.	L.Prakash
DBY747	MATa, trp1-289, ura3-52, leu2, his3.	R.H.Schiestl
DS1/38	MATα, leu2-3, leu2-112, his4, ura3-52.	D. Steele
TH4	MATα/MATa, his3-Δ1/HIS3, HIS4/his4, leu2-3/leu2-3, leu2-112/leu2-112, trp1-289/TRP1, ura3-52/ura3-52, rad18-2/RAD18.	This study
TH41-12	MATα/MATa, his3-Δ1/HIS3, HIS4/his4, leu2-3/leu2-3, leu2-112/leu2-112, trp1-289/TRP1, ura3-52/ura3-52, rad18-2/RAD18, DFR1/URA3:dfr1.	This study
TM12B	MATα, trp1-289, ura3-52, tup$^-$.	This study
TDBY747	MATa, trp1-289, ura3-52, leu2, his3, tup$^-$.	This study

to a final concentration of 250ug per ml). Isolation of dTMP-permeable yeast strains (tup mutants) was carried out on the MTX-SULF-supplemented YPDP medium in which the YPD medium was supplied with 1.5g KH_2PO_4 per liter at 34°C (Little and Haynes, 1979). The presporulation and sporulation media were those described by Sherman et al. (1974). Buffered synthetic selective medium used for assay of beta-galactosidase was described by Rose and Botstein (1983). Solid media was by addition of 2% Bacto agar. Unless stated otherwise, all components were sterilized by autoclaving for 20 minutes. To prepare buffered synthetic selective medium for beta-gal assay, salts and agar with sugar were brought to double strength and autoclaved separately. After cooling to 55°C, the two solutions were mixed, and the vitamins and 5-bromo-4chloro-3-indolyl-beta-D-galactosidase (X-gal) were then added. Soft agar (regeneration agar or top agar) was made by adding Bacto agar to liquid media to a final consentration of 0.6%.

IV. Yeast Genetic Methods

Yeast mating, sporulation and random spore analysis were carried out as described by Sherman et al.(1974).

The diploid strain TH4 was constructed by mating strain LP2729-4B with strain DS1/38 and isolating the diploid through complemention. Both haploid strains were grown on appropriate synthentic selective media and then single colonies were streaked on YPD medium. The streaks from each strain were perpendicular so that the mating of two strains occured at the

intersections. After 24 hr growth at 30°C, the YPD plate was replica plated on to omission plates. Several colonies growing in the intersections of the two haploids were picked and substreaked on another fresh SD plate for pure colony isolation. Before use in the gene disruption experiments, several colonies were tested to to confirm the ability of the diploid strain to sporulate.

For sporulation, a single colony from selective SD plate was transfered to presporulation medium. After two days, growth cells at 30°C were transfered to a sporulation plate for another two days. Sporulation was monitored by microscrope. For random spore analysis of the diploid strain TH41-12 containing a heterozygous disruption at the DFR1 locus, cells from the sporulation medium were suspended in 0.2ml of a 1/40 dilution of Glusulase and incubated with shaking for 1 to 2 hours. The treated asci were diluted to 5ml followed by sonication for about 3 to 5 minutes. Separation of spores from asci was monitored with a microscrope. Spores were judged to be separated adequately when greater than 95% of the spores were released from the asci. After appropriate several dilutions, 0.2ml of each dilutions were plated onto YPD plates. Emergent colonies were then picked and spotted on various selective plates for determination of phenotype.

V. Selection of dTMP-Permeable Strains

dTMP-permeable strains (tup mutants) were isolated as described by Little and Haynes (1979). YPDP selection plates containing SULF (5mg/ml) and MTX (100ug/ml) were supplemented

with dTMP (100ug/ml). Exponential cells were washed in 0.1M phosphate buffer (pH7.0) and plated to a final density of about $2x10^7$ cells/plate. After 5 days incubation at 34°c, the biggest colonies were picked and retested. Tup mutants thus isolated were used in the gene disruption and gene fusion studies.

VI. E.coli Transformations

The transformation of E.coli was done according to the calcium chloride method of Mandel and Higa (1970). Competent cells were used immediately in transformation experiments or stored at 4°C for up to 24 h before use. For routine transformations, cells were spread on LB plates supplemented with appropriate antibiotics. Plates were then incubated at 37°C overnight. Transformants were then retested for antibiotic resistance. For M13 bacteriophage transductions, cells were plated with soft agar onto the L plates, according to the procedure described by Pharmacia, Inc..

VII. Yeast Transfections

The transfection of yeast was carried out essentially as described by Hinnen et al.(1978) with modifications reported by McNeil et al.(1980). Either glusolase or zymolyase was used to prepare spheroplasts. Putative transfectants were scored after incubation for 3-5 days at 30°C. Transformants were then picked and retested on identical selection plates to confirm their phenotype.

XIII. DNA Purifications

Rapid isolation of covalently closed circular plasmid DNA from E.coli for use in restriction map analysis as well as bacterial and yeast transformations was done by the alkaline lysis procedure described by Birnboin and Doly (1979). The DNA samples were stored in TE buffer (pH8.0) which contained 10mM Tris-HCl, 1mM EDTA. If the sample was to be used for restriction map analysis, it was first treated for 15 minutes at room temperture with DNase-free RNase. The DNA sample could be stored at - 20°C for up to two days. A 1.5ml culture usually yielded about 1 to 2.5 ug plasmid DNA.

If a purer plasmid DNA preparation was required, a large scale purification was performed according to the cesium chloride density gradient procedure described by Maniatis et al.(1982). Plasmid DNA samples purified by this method could be stored at 4°C for at least one year without loss of biological activity.

Bacteriophage (RF) DNA preparation was conducted as described by Barnes et al.(1983). This procedure is basically similar to the large scale preparation for plasmid DNA except that bacterial cultures were first grown in LB medium to exponential phase and then infected with phage suspension. The infected culture was used to prepare the RF DNA by the method described above for a large scale preparation of plasmid DNA.

Single-stranded viral DNAs from recombinant M13mp18 and M13mp19 phages were prepared according to the manual for M13 cloning and sequencing provided by P-L Biochemicals,Inc. with minor modification. DNA samples were extracted sequentially with

phenol, chloroform/phenol, and then chloroform alone.

IX. DNA Manipulations

(i) Restriction Endonuclease Digestion. Plasmid DNA digestion with restriction endonucleases was performed in appropriate digestion buffers as described by Maniatis et al., (1982) or as recommended by the manufacturers.

(ii) Ligation of DNA Fragments. Ligation of DNA fragments was carried out in ligation buffer (50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP) with T4 DNA ligase. For routine ligation reactions (in a 20ul volume), 1 unit of the T4 DNA ligase was used for fragments with sticky ends while 5-10 units was employed for blunt ended ligations. Attachment of synthetic phosphorylated linker (50ug/ml) to fragments with blunt ends was performed as described for blunt-ended ligation. Ligation mixtures were incubated at 14°C for 6 to 24 hrs.

(iii) Filling Recessed 3' Ends of Double-stranded DNA. Filling recessed 3' ends of double-stranded DNA was achieved in 20ul nick-translation buffer (50mM Tris-HCl ph7.2, 10mM $MgSO_4$, 0.1mM dithiothreitol, 50ug/ml bovine serum albumin) containing 1mM dNTPs and 1 unit of Klenow fragment of E.coli polymerase I for 30 minutes at 20°C. The reaction was stopped by addition of 1ul of 0.5M EDTA.

(iv) Deletion of DNA Fragments with Bal31. Deletion of DNA fragments was accomplished in exonuclease Bal31 buffer containing 12mM $CaCl_2$, 12mM $MgCl_2$, 400mM NaCl, 20mM Tris-HCl pH8, 1mM EDTA and BSA (250ug/ml). As monitored on agarose gels, 1 unit of the

Bal31 (from BRL) removed about 100 bases from each end of the mixed fragments when about 7ug of the plasmid pIUD digested with either restriction enzyme HindIII or EcoR1 in 60 seconds. Bal31 reactions were stopped by addition of EDTA to a final concentration of 25mM.

(v) Quantitation of DNA. For quantitation of DNA samples the minigel method was used (Maniatis et al., 1982). The quantity of DNA was estimated by comparing with a DNA standard. For DNA samples purified by the large scale procedure, a spectrophotometric method was also utilized and the ratio of optical densities at 280 and 260 um was used as a measure of purity. The biological activity of the DNA sample was determined by its bacterial transformation frequency.

X. Agarose Gel Electrophoresis

Agarose gels were prepared with TBE buffer (89mM Tris-borate, 89mM boric acid and 2mM EDTA). The same buffer was employed during electrophoresis. DNA samples were loaded in the buffer described by Maniatis et al.(1982). Gels were stained with ethidium bromide and DNA was visualized with UV light in a Chromato-Vue Transilluminator (Model TM-15). Photography of the stained gel was conducted by using Polaroid High Speed 4x5 land film (Type 57). This general method of agarose gel electrophoresis was used for restriction map analysis of recombinant plasmids, identification of DNA fragments, and separation and purification of DNA fragments required for the constructions of

various recombinant plasmids.

XI. Recovery of DNA from Agarose Gels

The electroelution method described by Maniatis et al.(1982) was adopted. After separation of a particular fragment from the others in agarose gels after electrophoresis, a gel slice containing the band of interest was cut out and placed into a dialysis membrane filled with TBE buffer. The DNA was then electroeluted from the gel slice into the dialysis bag in an electrophoresis tank. DNA in the solution was thus purified by phenol/chloroform extraction and recovered by ethanol precipitation.

XII. Plasmid Constructions

Vectors and plasmids used or constructed during the course of this study are given in Table 3. Plasmid pIUD-1 (constructed by M.Nagel in our laboratory) was used as a donor of the cloned yeast genomic Sal1/BamH1 restriction fragment, containing the DFR1 gene. It was also employed in the constructions of DFR1:LacZ fusions. Plasmid pLG669-Z (Guarrente and Ptashne, 1981) was used in the construction of the DFR1:LacZ fusions and was the donor of the HindIII restriction fragment containing the yeast URA3 gene. Both plasmid pSV2-neo (obtained from BRL) and pYF91 (obtained from J.D. Freisen's lab. University of Toronto) were employed in the construction of plasmids used in gene disruption experiments. Plasmid pND21 (constructed by V.

Table 3. Vectors and Plasmids used in this study

Plasmid	Characteristics	Source/ref.
pIUD1	ori, Ap^r, URA3, DFR1.	M.G. Nagel
pLG669-Z	ori, Ap^r, URA3, 2u, CYC1:lacZ fusion.	L.Guarrente
pSV2-neo	ori, Ap^r, SV40ori, neo .	BRL.
pYF91	ori, Ap^r, 2u, LEU2.	J.D.Freisen
pDN21	ori, Ap^r, SV40ori, neo , DFR1.	V. Barclay
Yip5	ori, Ap^r, URA3, Tc .	J.D.Freisen
pNHO-1	ori, Ap^r, SV40ori, neo .	This study
pNBD-2	ori, Ap^r, SV40ori, neo , DFR1.	This study
pNBU-3	ori, Ap^r, SV40ori, neo , URA3:dfr1.	This study
pDEH-5	ori, Ap^r, URA3, Tc^r, dfr1 (Δ5',3').	This study
pLBB20	ori, Ap^r, 2u, LEU2, DFR1.	This study
pHBB200	ori, Ap^r, URA3, dfr1 (Δ3').	This study
pDSB1-Z	ori, Ap^r, 2u, URA3, DFR1, 'lac-Z.	This study
pEFZ-1	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion	This study
pEFZ-2	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-3	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-4	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-5	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-6	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-7	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-8	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study

Table 3. (cont'd)

Plasmid	Characteristics	Source/Ref.
pEFZ-9	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-10	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pEFZ-11	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-1	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-2	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-3	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-4	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-5	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-6	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-7	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-8	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-9	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-10	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-11	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-12	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-13	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-14	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-15	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-16	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-17	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study
pHFZ-18	ori, Ap^r, 2u, URA3, DFR1:lacZ fusion.	This study

Table 3. (cont'd)

Plasmid	Characteristics	Source/Ref.
M13mp8B13 H	HindIII/BamH1 fragment in M13mp8	M.G.Nagel
M13mp19EH	EcoR1/HindIII fragment in M13mp19	This study
M13mp19EB	EcoR1/BamH1 fragment in M13mp19	This study
M13mp18HS	HindIII/Sal1 fragment in M13mp18	This study
M13mp19SH	Sal1/HindIII fragment in M13mp19	This study
M13mp18F10	149bp Sau3A fragment (5'to 3') in mp18	This study
M13mp18F12	149bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18F19	260bp Sau3A fragment (5'to 3') in mp18	This study
M13mp18F52	260bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18S3	60bp Sau3A fragment (5'to 3') in mp18	This study
M13mp18F8	60bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18F6	105bp Sau3A fragment (5'to 3') in mp18	This study
M13mp18F3	105bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18F11	60+128bp Sau3A fragments (5'to 3') in mp18	This study
M13mp18F4	128bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18F9	139bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18F37	525bp Sau3A fragment (5'to 3') in mp18	This study
M13mp18F22	525bp Sau3A fragment (3'to 5') in mp18	This study
M13mp18S6	238bp Sau3A fragment (5'to 3') in mp18	This study
M13mp18F38	238bp Sau3A fragment (3'to 5') in mp18	This study

Barclay in this laboratory) was a donor of the BamH1 restriction fragment containing the DFR1 gene. Plasmid Yip5 was used as a vector for insertion of an EcoR1/HindIII restriction fragment of the internal fragment within the coding regiion of DFR1 gene.

The following plasmids were constructed during the course of gene disruption experiments. Plasmid pNHO-1 was derived from pSV2-neo by filling in its unique HindIII site. Plasmid pNBD-2 was then constructed by an insertion, into the unique BamH1 site of pNHO-1, of a BamH1 restriction fragment containing DFR1, isolated from plasmid pDN-21. Plasmid pNBU-3 was obtained by insertion of a HindIII restriction fragment containing a URA3 gene, isolated from plasmid pLG669-Z, into the unique HindIII site of the plasmid pNBD-2. Plasmid pDEH-5 was produced by replacement of its smaller EcoR1/HindIII restriction fragment with an internal sequence within the coding region of the DFR1. Plasmid pLBB20 was derived by insertion of a BamH1 restriction fragment containing DFR1, into the unique BamH1 site of plasmid pYF91.

To construct the pEZF plasmids, approximately 7 ug of plasmid pIUD-1 DNA was first digested with 30 units of EcoR1 restriction enzyme at 37°C for two hours. The DNA sample was then extracted with phenol to move proteins and recovered with ethanol. The DNA pellet was dissolved in 100 ul of 500ug/ml bovine serum albumine solution and 100 ul Of 2 x Bal31 buffer (24mM $CaCl_2$, 24mM $MgCl_2$, 800mM NaCl, 40mM Tris-HCl(pH8.0), 2mM EDTA) to a final concentration of about 33ug DNA/ml. One unit of exonuclease Bal31 (BRL) was added to the solution and the

reaction mixture was incubated at 25°C. The extent of the reaction was monitored by running a small amount of sample on 0.7% agarose gel. Under these conditions, the enzyme removed approximately 100 bp from each end per minute. After 30 and 45 seconds of incubation, 100 ul aliquots were removed from the reaction mixture and the Bal31 reaction was terminated by addition of EDTA to a final concentration of 25mM. The samples were pooled and the DNA was then extracted with phenol and precipitated with ethanol. The recessed 3'-ends of the double-stranded DNAs which were left after treatment with Bal31 were repaired in 20 ul of Klenow fragment buffer by reaction with one unit of Klenow fragment of E.coli DNA polymerase I at 25°C for 30 min. The reaction was stopped by addition of 1 ul of 500mM EDTA. After phenol extraction and ethanol precipitation, attachment of synthetic oliganucleotide Bam linker onto the blunt ends was carried out in 20 ul of ligation buffer. 1.5 ug of phosphorylated BamH1 linker (BRL) and two units of T4 DNA ligase were then added. The mixture was incubated at 14°C overnither. After phenol extraction and ethanol precipitation, about 5 ug of the DNA sample was digested with excess amount of restriction enzyme BamH1 (200 units) for 5 hours to ensure the generation of the BamH1 sticky ends from the attached Bam linker. Proteins were again removed by phenol extraction and the DNA sample was recovered by ethanol. Digestion with restriction enzyme Sal1 was then performed to release the novel Sal1/BamH1 fragments in which the 3' end of the coding region of the DFR1 gene had been deleted. The fragments of interesting fragments were then

separated from the linker and other DNA fragments by agarose gel electrophoresis and purified by electroelution. One-third of the recovered DNA was used to ligate into the purified Sal1/BamH1 restriction fragment of pLG669-Z. This ligation mixture was then used to transform the E.coli LacZ mutant strain MC1064. Transformants with potential fusion plasmids were selected on Amp-LB agar plates containing X-gal.

The procedure used in construction of pHFZ fusion plasmids was basically same as in the construction of the pEFZ fusion plasmids described above, except that plasmid pIUD-1 was first digested with restriction enzyme HindIII rather than with EcoR1. This was to make the deletion end points much closer to the translation initiation site of the DFR1 gene than those in the pEFZ fusions. For the similar reasons, the reaction time of Bal31 digestion was increased to between 45 and 60 seconds, to come close to the terminus of the DFR1 cofing region. In addition, after attachment of the BamH1 linker and complete digestion with restriction enzyme BamH1 to generate the BamH1 sticky ends from the attached BamH1 linker, the fragments of interesting were separated by agarose gel electrophoresis and purified from the gel. The molecules were then recircularized in a ligation reaction and amplificated in E. coli. Over 200 colonies were pooled and plasmid DNAs (pHBB200) from this mixed culture were then purified by the large-scale procedure. These changes provided much more of the Bal31-generated DFR1' fragments which greatly facilitated the final construction of pHFZ fusions. The original cloned Sal1/BamH1 genomic DNA fragment

containing the intact DFR1 gene was also inserted into the vector pLG669-Z (resulting in plasmid pDSB1-Z) for use as a contral plasmid in fusion experiments.

Bacteriophage M13mp18 and M13mp19 were used in subcloning of the yeast DNA fragment to be sequenced. Plasmids constructed and used during the course of sequencing the cloned Sal1/BamH1 yeast genomic DNA fragment are summarized in Table 3. The Sal1/HindIII segment was cloned in both orientations into both M13 vectors. The EcoR1/HindIII and EcoR1/BamH1 segments were force-cloned into M13mp19. The other contructions were obtained by "shotgun" cloning a pool of Sau3A restriction segments generated by the digestion of the purified Sal1/BamH1 segment with restriction enzyme Sau3A into M13mp18.

XIII. Analysis of Recombinant DNAs

Characterization of recombinant plasmids was carried out in two ways. in vivo approaches were used if recombinant plasmids were able to confer a different phenotype upon their bacterial host (ie. resistance to antibiotics, and prototrophy or auxotrophy for certain nutrients). In the case of Tm resistance, bacterial transformants were first selected as Amp resistant. Emergent colonies were then transfered to M9 selection plates to score for Tm resistance. Once a potential recombinant plasmid was identified by this in vivo approach, it was then analysized by restriction enzyme digestion and agarose gel electrophoresis.

M13 recombinant molecules containing insert fragments for sequencing were recognized as clear plaques on agar plates

containing IPTG (Isopropyl-beta-D-thiogalactopyranoside) and X-gal as described by Pharmacia. Size of the inserts was determined by analysis of the migration of the single stranded viral DNA (ssDNA) derived from clear plaques on agarose gels. M13mp18 or M13mp19 ssDNA was used as standard. In the case of M13 recombinants derived from Sau3A shotgun cloning, the orientations of the inserts was determinated by the complementation (C-test) on agarose gels according to procedure described by Barnes et al. (1983).

XIV. DNA Sequencing

The Sanger dideoxy chain termination method (Sanger et al., 1977) was utilized in determination of the nucleotide sequence of the cloned yeast genomic DNA. The hybridization of primer and template, and the dideoxy chain termination reactions were performed essentially as described in the M13 cloning/sequencing manual suppled by P-L Biochemicals, Inc.. The products of the sequencing reactions were denatured by heating by boiling for 3 min. and resolved on 8% polyacrylamide/8M urea gels with running times of 2 and 8 hours. After the electrophoresis was completed, the gel was exposed to Kodak XAR-5 film at -70°C for 1 to 3 days. The film was developed in Kodak GBX developer for 2 to 5 min. and fixed in Kodak GBX fixer for 5 min.. Base sequences were read directly from the autoradiographed film. A computer program (U of MINU. Sequence Analysis Programs, Version 2.1, 1982) was used for storage and analysis of the DNA sequence data.

XV. Beta-galactosidase Assay

(i) Plate assays. Bacterial transformants containing putative fusion plasmids were spotted onto LB-Amp agar plates or M9-lactose agar plates, containing either 40ug/ml or 200ug/ml of X-gal. Yeast transformants were selected on SD medium and, subsequently, spotted on buffered SD medium (pH7.0) containing 200ug/ml of X-gal. For more sensitive detection of beta-galactosidase activities in yeast colonies, the filter permeabilizing method suggesed by Casadaban et al. (1983) was used.

(ii) Culture assay. Bacterial assay for beta-galactosidase activity was performed as described by Miller (1972). Yeast transfectants were assayed by two methods. The permeabilized cell method employed was essentially as described by Guarente and Ptashne (1981) with the modifications suggested by Ruby et al. (1983). The crude cell lyzate method used was that described by Rose et al. (1981). Protein was measured by the Lowry method.

RESULTS

Before this study began, the yeast DHFR gene (DFR1) had been isolated on an 8.8 kb Bam H1 restriction fragment from the J. Freisen yeast genomic library pYF94 by selection of E.coli transformants resistant to trimethoprim (Tm) (Barclay et al., manuscript in preparation). The gene was subsequently localized on an 1.7 kb Sal1/Bam H1 restriction fragment which when cloned in either orientation into multicopy or integrating vectors resulted in increased MTX resistance in yeast. The degree of drug resistance was dependent upon the gene copy number (see Fig.2). Further evidence that this fragment encoded the yeast DHFR was obtained by G. Wahl who showed that plasmids containing the putative cloned DFR1 gene complemented an E.coli DHFR- deficient mutant strain (G. Wahl, personal communication). These observations suggested that the cloned 1.7 kb Sal1/BamH1 restriction fragment contained the yeast DHFR structural gene and regulatory elements, which allowed for DFR1 expression both in E.coli and in yeast. Hence, this restriction fragment was used in more detailed investigations of the structure and regulation of the yeast DFR1 gene.

I. DNA Sequence Analysis of the Sal1/BamH1 Restriction Fragment Containing the Yeast DFR1 Gene

The DNA sequence of a gene provides useful information about the physical properties of the gene product, such as, the amino acid sequence and molecular weight of the encoded polypeptide. In addition, such sequencing information can be used to identify

Figure 2 DFR1-mediated resistance to antifolates

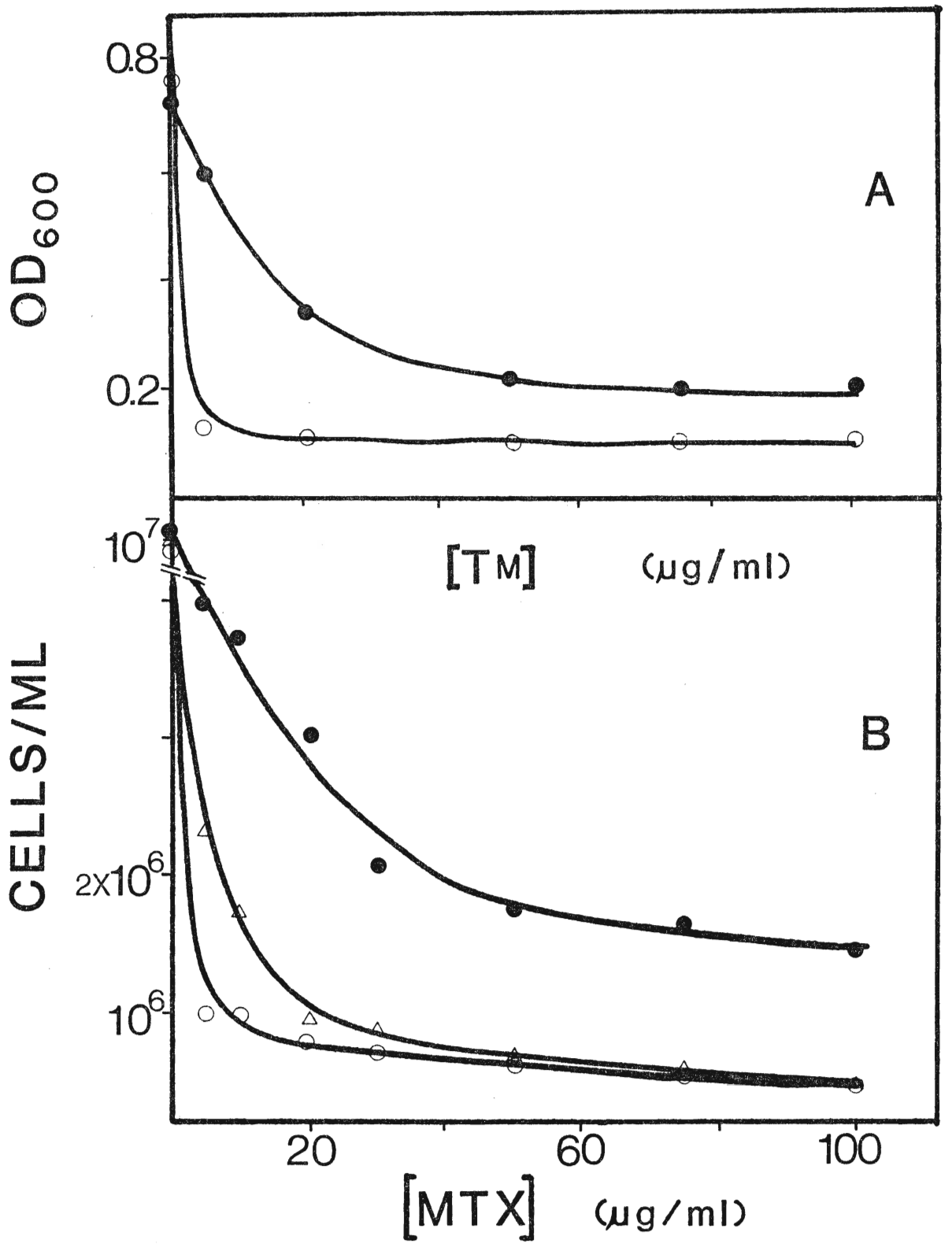
(A). ○: JF1754/pLG669-Z
●: JF1754/pDSB1-Z

Bacterial cells harboring the plasmids were grown in LB medium containing 50ug/ml of Amp, for overnight. Cells were washed once with M9 medium and resuspended, at a cell density of about 0.2 of OD at 600nm, in the M9 media containing Met, His, Leu and various consentrations of Tm (0, 5, 20, 50, 75 and 100ug/ml). After 24h subsequent culture, the growth was indicated by OD values at 600nm.

(B). ○: M1/2B/pLG669-Z
△: M1/2B/pIUD1
●: M1/2B/pDSB1-Z

Yeast cells containing the plasmids were grown in SD medium containing Trp (20ug/ml), to about 10^6 cells/ml. Exponential cells were harvested by centrifugation and resuspended in SD medium supplemented with SULF (5mg/ml) and MTX at various concentrations. After 24h incubation at 30°C, the cells densities were determined by a Coulter counter.

Structure of the plasmids is shown in Fig. 10.


0.8
0.2
OD600
A
[TM] (µg/ml)
10^7
2X10^6
10^6
CELLS/ML
B
20
60
100
[MTX] (µg/ml)

potential regulatory elements continguous to the coding region by comparison of these sequences with canonical structures identified from other genes as having some important function in gene expression. Besides providing useful information about the gene product, which can be obtained directly from the sequencing data, more importantly, such detailed information is extremely helpful in the construction of various molecular tools to be used and the design of experiments to investigate gene function and regulation at the molecular level.

Modern methods for DNA sequencing fall into two broad catagories: the primed synthesis method developed by Sanger et al. (1977) and the chemical method developed by Maxam and Gilbert (1977). The first method employs a primed DNA synthesis approach in which a single stranded template which contains the sequence of interest is copied to produce a radioactively labelled complementary strand. By the use of the chain-terminating inhibitors, 2',3' dideoxynucleoside 5' triphosphates, sets of partially elongated molecules are produced, which can be fractionated on denaturing electrophoresis gels. The patterns of labelled bands obtained are then used to deduce the primary base sequence. Highly efficient procedures have been developed for the generation of single stranded templates from any cloned duplex DNA utilizing the bacteriophage M13, which has given rise to a broad adoption of this method for DNA sequencing. Because of the ease with which the system can be utilized, the Sanger dideoxy chain terminator methodology combined with the M13 cloning system has become increasingly popular in recent years.

Some sequencing data of the DFR1 DNA fragment had been obtained previously by Michael Nagel in our laboratory (M. Nagel M.Sc. Thesis, Brock University, 1985). However, the data obtained by the beginning of this study were somewhat limited. Thus, to facilitate further studies on DFR1 gene regulation, I began this study by sequencing the Sal1/BamH1 restriction fragment containing the DFR1 gene in its entirety.

A. Nucleotide Sequence of the Sal1/BamH1 Restriction Fragment Containing the DFR1 Gene

The strategy employed in sequencing the DFR1 gene is shown in Fig. 3. The nucleotide sequence data shown in Fig. 4 are the combined results from the analysis of various segments of the Sal1/BamH1 restriction fragment. As shown, within the fragment which comprises a total of 1784 bases is a single open reading frame of 633 bp, extending from residue 800 to 1432. This coding region is sufficient to encode a polypeptide of 211 amino acids, with a predicted molecular weight of 24,229.8 Daltons. The region proximal to the ATG codon, tentatively assigned as the translation initiation site of the DFR1 gene (at position 800), is in good agreement with that suggested as the prefered environment of translation initiation sites of many eukaryotic genes (Dobson et al., 1982; Kozak, 1984), i.e., an A is usually found at position -3, a G at +4 (yeast) and a T at +6 (higher eukaryotes) with respect to the ATG codon at position 1. Also consistent with this assignment is the finding that the sequence upstream of the ATG codon shows several features of promoter

Figure 3 Sequencing strategy for the 1.7 kb DFR1 DNA fragment

M13 cloning was as described previously in Methods. The horizontal lines with arrowheads indicate the length and direction of the sequenced M13 clones. The extent of the DFR1 open reading frame is indicated at the top of the fiqure. Abbreviations for restriction sites are as following: B, BamH1; P, Pst1; E, EcoR1; Pv, PvuII; H, HindIII; S, Sal1; sa, Sau3A.

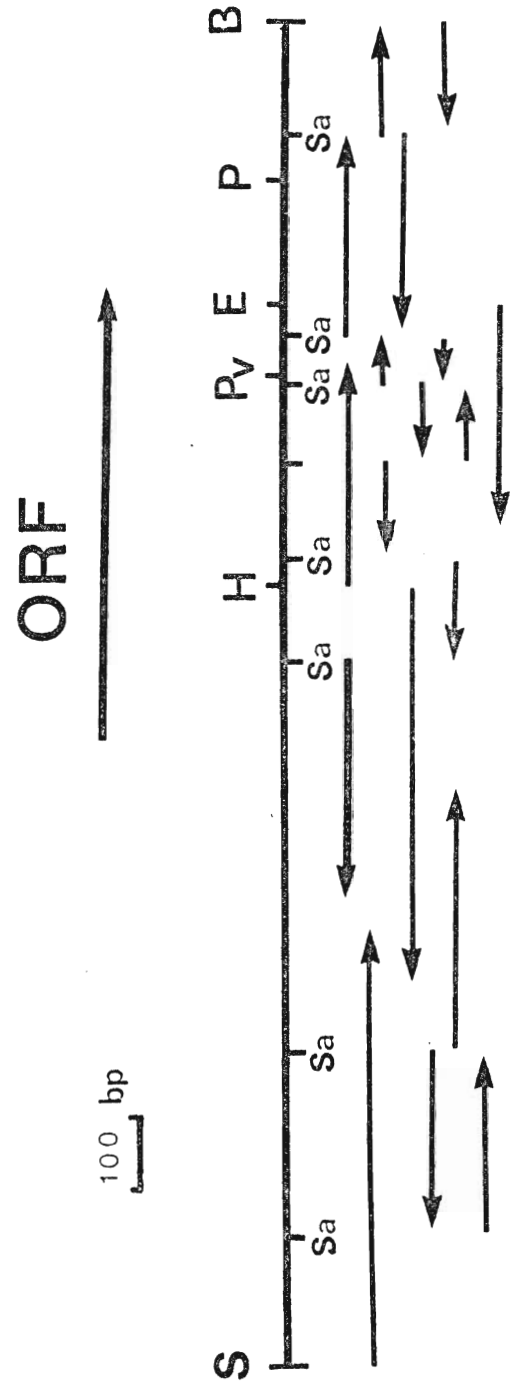

ORF
100 bp
S
Sa
Sa
Sa
H
Sa
Sa
Pv
E
P
B

Figure 4. Nucleotide and predicted amino acid sequence of the DFR1 gene

Potential "TATA" boxes are underlined. Homologous sequences with the core sequence required for general amino acid control are marked by wave underline.

TRANSLATION OF DFR1

```
   2
TCGACGTACTTCAGTATGTAATATACCCCAAACATTTTACCCACAAAAAACCAGGATTTGAAAACTATAGCATCTAAAAG

  82
TCTTAGGTACTAGAGTTTTCATTTCGGAGCAGGCTTTTTGAAAAATTTAATTCAACCATTGCAGCAGCTTTTGACTAACA

 162
CATTCTACAGTAGGATCATTTCTATAGGAATCGTCACTCTTTGACTCTTCAAAAGAGCCACTGAATCCAACTTGGTTGAT

 242
GAGTCCCATAACCTTTGTACCCCAGAGTGAGAAACCGAAATTGAATCTAAATTAGCTTGGTCCGCAATCCTTAGCGGTTC

 322
GGCCATCTATAATTTTGAATAAAAATTTTGCTTTGCCGTTGCATTTGTAGTTTTTTCCTTTGGAAGTAATTACAATATTT

 402
TATGGCGCGATGATCTTGACCCATCCTATGTACTTCTTTTTTGAAGGGATAGGGCTCTATGGGTGGGTACAAATGGCAGT

 482
CTGACAAGTTAACCACTTTTTTCCTTTTCTAAATTGTTTAAAACCAAAGGTTTGGTTTTCAGTTAAGAAATTGGATTAGT

 562
TGGTGTGTAAGTATAATTAAATGTAGTCTTGGTTAGCTTAATGTATAGGTTCTTGATAGTATGACTCCACCTAAAATTCG

 642
TCAAGAGCGACGGATGCGAAAACAAAAAAATCGCTTTGCATCCTTACGTGCGTGAGATGGCATCGCTGGCCAAAGGGAAA

 722
CGTTTCATTATTTTCTGTAGTTAACATTATGCTTTGCATGATAATAAGAGAAATTGAAGAGCGCAACGAACTACGAGC

 800
MET ALA GLY GLY LYS ILE PRO ILE VAL GLY ILE VAL ALA CYS LEU GLN PRO GLU MET GLY
ATG GCT GGA GGA AAG ATT CCT ATT GTA GGA ATT GTG GCA TGT TTA CAG CCG GAG ATG GGG

 860
ILE GLY PHE ARG GLY GLY LEU PRO TRP ARG LEU PRO SER GLU MET LYS TYR PHE ARG GLN
ATA GGA TTT CGT GGA GGT CTA CCA TGG AGG TTG CCC AGT GAA ATG AAG TAT TTC AGA CAG

 920
VAL THR SER LEU THR LYS ASP PRO ASN LYS LYS ASN ALA LEU ILE MET GLY ARG LYS THR
GTC ACT TCA TTG ACG AAA GAT CCA AAC AAA AAA AAT GCT TTG ATA ATG GGA AGG AAG ACA

 980
TRP GLU SER ILE PRO PRO LYS PHE ARG PRO LEU PRO ASN ARG MET ASN VAL ILE ILE SER
TGG GAA TCC ATA CCG CCC AAG TTT CGC CCA CTG CCC AAT AGA ATG AAT GTC ATT ATA TCG
```

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1040
ARG SER PHE LYS ASP ASP PHE VAL HIS ASP LYS GLU ARG SER ILE VAL GLN SER ASN SER
AGA AGC TTC AAG GAC GAT TTT GTC CAC GAT AAA GAG AGA TCA ATA GTC CAA AGT AAT TCA

1100
LEU ALA ASN ALA ILE MET ASN LEU GLU SER ASN PHE LYS GLU HIS LEU GLU ARG ILE TYR
TTG GCA AAC GCA ATA ATG AAC CTA GAA AGC AAT TTT AAG GAG CAT CTG GAA AGA ATC TAC

1160
VAL ILE GLY GLY GLY GLU VAL TYR SER GLN ILE PHE SER ILE THR ASP HIS TRP LEU ILE
GTG ATT GGG GGT GGC GAA GTT TAT AGT CAA ATC TTC TCC ATT ACA GAT CAT TGG CTC ATC

1220
THR LYS ILE ASN PRO LEU ASP LYS ASN ALA THR PRO ALA MET ASP THR PHE LEU ASP ALA
ACG AAA ATA AAT CCA TTA GAT AAA AAC GCA ACT CCT GCA ATG GAC ACT TTC CTT GAT GCC

1280
LYS LYS LEU GLU GLU VAL PHE SER GLU GLN ASP PRO ALA GLN LEU LYS GLU PHE LEU PRO
AAG AAA TTG GAA GAA GTA TTT AGC GAG CAA GAT CCG GCC CAG CTG AAA GAA TTT CTT CCC

1340
PRO LYS VAL GLU LEU PRO GLU THR ASP CYS ASP GLN ARG TYR SER LEU GLU GLU LYS GLY
CCT AAA GTA GAG TTG CCC GAA ACA GAC TGT GAT CAA CGC TAC TCG CTG GAA GAA AAA GGT

1400
TYR CYS PHE GLU PHE THR LEU TYR ASN ARG LYS @@@ AACCTCTCCGCCCGTATATTTTTTTAATAT
TAT TGC TTC GAA TTC ACT CTA TAC AAT CGT AAA TGA

1467
GTTAAATAGTGATAGAACTGATAAGCCTCATTTTCTTTTATTGGGCTCCAAGACGCGAACTGTTCGTAGGGTAACCGTTT

1547
GAGACCTAAACGACCTTTCAGCCTCACCTGCAGTATTTCTTCAACAACGCCTGTCGCTATGTTAAATAATAGCAATCGTT

1627
TGTGATCACCATTGTCGAATTTGACGCGCTTAAACCAAAAACCATTGTTTTGGCCTCGTTCCCTGCATTCAACAAAAGAG

1707
CAAGGTATGCCGTCAAACAGTCGTTAAAAGAGAAGGTTTATAAACTATCTTGTTTTGTACTTTGCTGTCCCGGATCC


1784


THE MOLECULAR WEIGHT OF THIS PROTEIN IS  24229.8
```

elements found in other yeast genes (see Results I, Section C). Several sequences downstream of the putative translational termination codon TGA (at position 1433) are in good agreement with those which are highly conserved in several transcription termination sequence elements in yeast (see Results I, Section C). A comparison of the amino acid sequence deduced from the assigned DFR1 coding region with those of DHFR proteins determined previously from various organisms reveals that conserved sequences are located throughout the polypeptide (see Results I, Section E). Further evidence that this assigment was correct was obtained in experiments described later in this study (see Results V), in which a HindIII restriction fragment containing the functional yeast URA3 gene was inserted into an analogous restriction site within the putative coding region. A host bacterium containing this plasmid construction remained sensitive to Tm. This result suggested that the DFR1 had been disrupted by the URA3 DNA, consistent with my putative assignment of the DHFR coding region. Furthermore, functional expression in yeast of the two classes of DFR1:lacZ fusions constructed later in this study is also in agreement with such an assignment.

C. Regulatory Sequences of the DFR1 Gene

Most eukaryotic regulatory sequences located 5'to structural genes can be divided into several distinct regions based upon their presumed function during gene expression. These include the transcriptional initiation site (I), the "TATA" box and upstream

regulatory elements (Benoist and Chambon, 1981; Guarente,1984; Serfling et al.,1985). In multicellular eukaryotes, the "TATA" box is thought to direct the eukaryotic polymerase II to initiate transcription at a discrete site, which is usually located about 30 bases downstream. In most cases, there seems to be no other specific sequence requirements at this site for transcriptional initiation (I site). In common with higher eukaroyotic cells, a "TATA" box has been found in the 5′ flanking sequences of most yeast genes examined to date. However, in contrast to observations made in multicellular eukaryotes, the distance between the yeast "TATA" box and the I site is quite variable, ranging from 35 to 180 nucleotides (Sentenae and Hall, 1982). Moreover, it has been shown that transcriptional initiation in yeast often occurs at or near a sequence idential or closely related to "CAAG" or "TCAA". In addition, a C-T rich block is usually found upstream of the "CAAG" sequence in genes which are expressed constitutively at a high level (Holland and Holland, 1980; Montgomery et al,1980; Dobson et al.,1982; Burke, 1983; Hahn et al.,1985).

A search for these canonical sequences in the 5′ flanking region of the DFR1 gene identified three potential "TATA" boxes at position -473, -227 (TATAAT) and -37 (TAATAA) with respect to the assigned translational initiation site. The canonical sequences "TCAAG" occurs 63 bp downstream of the "TATAAT" sequence. An interesting feature of this sequence is that it represents an overlap of the canonical regulatory signals "TCAA" and "CAAG". Another "CAAG" sequence apperaes downstream of

the sequence "TATAAT" (at -473). Several of the yeast consensus sequence "CAAG" are found immediately downstream of the other TATA-like sequence "TAATAA". However, in contrast to several other yeast genes, no large C-T rich blocks were observed between either of these putative "TATA" boxes and the "CAAG" sequences.

It is a characteristic feature of upstream regulatory elements of many eukaryotic genes that they can act in both orientations at long distances from the transcriptional initiation site (Guarente 1984; Serfling et al., 1985). In yeast, these elements have been designated as "upstream activation sites" (UASs), which resemble, in many respects, the "enhancer" sequences observed in higher eukaryotes. Study of a variety of yeast genes known to be involved in various pathways of intermediary metabolism has shown that UAS regions are defined by DNA sequences which interact with trans-acting factors encoded by unlinked regulatory genes. A search of the DFR1 DNA sequence obtained in this study for consensus with the core sequences of yeast UAS elements revealed two repeats of the sequence "TGACTC" (at position -177 and -597 bp upstream of the translational initiation codon). This UAS core sequence is thought to be necessary for the amino acid general control depression response (Jones and Fink, 1983; Lucchini et al., 1984) and as the binding target of the transcription factor GCN4 (Arndt and Fink, 1986). This finding suggests that the DFR1 might also be regulated by the general amino acid general control system. In support of this possibility, it is worthy of note that DHFR plays

a important role in the biosynthesis and metabolism of several amino acids such as methionine and histidine (Blakley, 1984) and that the latter amino acid has been shown to be subject to the amino acid general control in yeast.

In addition to the regulatory elements which can be identified in the DNA sequence upstream from the DFR1 coding region, several interesting features can also be observed in the sequence 3' to the gene. It has been suggested that there is a coupling of yeast RNA transcriptional termination and poly-adenylation (Zaret and Sherman, 1982). In spite of the fact that the precise molecular signals for these processes have yet to be identified, several putative transcriptional termination sequences have been proposed. For example, the tripartite consensus sequence (TAG...TAGT/TATGT....TTT) first observed by Zared and Sherman (1982) has since been found in many other yeast genes. In the case of the DFR1 gene, three sets of these matched sequences can be identified, (see Fig.5a; some of these observations have been obtained by M.G. Nagel, M.Sc. thesis, Brock University, 1985). In addition, Henikatt et al. (1983) have observed another sequence ("TTTTTATA") thought to be neccessary for efficient transcription termination in yeast and proposed to resemble the prokaryotic rho factor-dependent terminators. The DFR1 3' flanking sequence contains a closely related sequence (see Fig. 5b). An interesting feature of this region is that it also shares some features with the prokaryotic rho factor-indenpent terminators, namely, there are 8 consecutive T residues 22 bp downstream of the TGA stop codon, preceded by a GC

Figure 5. 3' consensus sequences of the DFR1 gene

(a)

Yeast: TAA/TAG/TGA.....1-140.....TAG.....TAGT/TATGT.....TTT...

DFR1: TGA.....31.....TATGT....28....TTT...
TGA.....40.....TAGT.....20....TTT...
TGA...40...TAG...3...TAG...51...TAG..68...TATGT....16....TTT...

(b)

YEAST: TAA/TAG/TGA.....TTTTTATA.....

DFR1: TGA.....24.....TTTTTAATA.....

(c)

YEAST: TAA/TAG/TGA.....TAAATAA(G/A).....

DFR1: TGA.....36.....TAAATAG.....
TGA.....176.....TAAATAATA.....

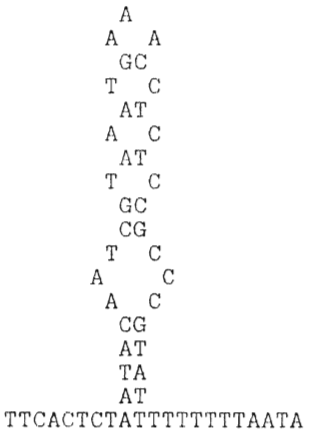
(d)

Eukaryote: TAA/TAG/TGA.....AATAAA.....

DFR1: TGA.....AAATAA.....

(E)

DFR1:



rich region forming a portion of a partial dyad symmetry (see Fig. 5e; Platt, 1981). Also found in the 3' non-coding region of the DFR1 gene are the sequences which very closely resemble the other consensus sequence "TAAATAA(G/A)" proposed by Bennetzen and Hall (1982) as important for yeast transcriptional termination and thought to be reminiscent of the canonical sequence "AATAAA" required for polyadenylation in higher eukaryotes (Fig. 5c). Still another sequence "AAATAA" located 175 bp downstream from the termination codon shows considerable homology with the sequence "AATAAA" postulated to be important for polyadenylation of eukaryotic mRNAs (Fig.5d, Proudfort and Brownlee, 1976; Benoist et al., 1980; this observation has also been pointed out by M.G. Nagel, 1985). Further experiments are necessary to determine with any certainty that any of these DNA sequences is responsible for the transcriptional termination and/or polyadenylation for the DFR1 gene in yeast.

D. Codon Usage of the DFR1 Gene

In yeast genes, the pattern of codon usage varies considerably (Bennetzen and Hall, 1982). A survey of the DNA sequences of the coding region of 110 yeast genes has confirmed a relationship between the level of expression of a particular gene and the degree of codon usage bias toward particular triplets, which is most extreme in highly expressed genes (Sharp, 1986). It has been demonstrated that genes which are efficiently expressed utilize preferentially only 25 codons out of the possible 61 coding triplets, whereas genes which are poorly expressed have a

less restricted pattern of codon usage (Benetzen and Hall, 1982). These studies have also revealed that for a given amino acid, the major isoaccepting tRNA species present in yeast possess anticodons corresponding to the most frequently used triplet, thus allowing for efficient translation of prefered codons.

Bennetzen and Hall (1982) have defined a codon bias index (CBI), on a scale of 0 - 1.0, that quantitates the extent of the bias towards a set of 22 prefered codons. Thus, when these 22 codons are used exclusively, the CBI is one, while it is zero when the usage of these triplets is exactly that expected if the codons were distributed randomly.

A summary of the codons used in the yeast DFR1 mRNA is presented in Table 4. The CBI of the yeast DHFR mRNA was 0.0083, calculated according to the rules suggested by Bennetzen and Hall (1982). This value is very close to zero and indicates almost totally random codon usage. This suggests that the DFR1 gene is expressed at a relatively low level under normal physiological conditions.

F. Comparison of Yeast DFR1 Amino Acid Sequence with Other DHFRs

During the last decade, X-ray crystallographic studies and amino acid sequencing have led to significant advances in our understanding of the structure of various DHFRs and the molecular basis of inhibitor and/or cofactor affinities for these proteins. To date, 12 different DHFR proteins have been completely sequenced. the predicted amino acid sequences deduced from the

Table 4. Codon usage of the DFR1 gene

AA	Cod	no. cod	no. aa	AA	Cod	no. cod	no. aa	AA	Cod	no. cod	no aa
Phe	UUU	6		Pro	CCU	3		Asn	AAU	7	
Phe	UUC"	6	12	Pro	CCC	5		Asn	AAC"	4	11
				Pro	CCA"	4					
Leu	UUA	2		Pro	CCG	3	15	Lys	AAA	11	
Leu	UUG"	6						Lys	AAG"	7	18
Leu	CUU	2		Thr	ACU"	4					
Leu	CUC	1		Thr	ACC"	0		Asp	GAU	8	
Leu	CUA	3		Thr	ACA	3		Asp	GAC	3	11
Leu	CUG	4	18	Thr	ACG	2	9				
								Glu	GAA"	12	
Ile	AUU"	6		Ala	GCU"	2		Glu	GAG	5	17
Ile	AUC"	3		Ala	GCC"	1					
Ile	AUA	7	16	Ala	GCA	5		Cys	UGU"	2	
				Ala	GCG	1	9	Cys	UGC	1	3
Met	AUG	7	7								
				Tyr	UAU	3		Trp	UGG	3	3
Val	GUU"	1		Tyr	UAC"	3	6				
Val	GUC"	4						Arg	CGU	2	
Val	GUA	3		Term	UAA	0		Arg	CGC	2	
Val	GUG	2	10	Term	UAG	0		Arg	CGA	0	
				Term	UGA	1	1	Arg	CGG	0	
Ser	UCU"	0						Arg	AGA"	5	
Ser	UCC"	2		His	CAU	2		Arg	AGG	2	11
Ser	UCA	3		His	CAC"	1	3				
Ser	UCG	2						Gly	GGU"	3	
Ser	AGU	3		Gln	CAA"	4		Gly	GGC	1	
Ser	AGC	3	13	Gln	CAG	3	7	Gly	GGA	6	
								Gly	GGG	2	12

Codons marked by " are those which were shown by Bennetzen and Hall (1982) to be selectively used more than 85% of the time in ADH1 and G3PDH genes. Cod = codons, AA or aa = amino acids.

corresponding DNA sequences of 11 DHFR genes are also available (see appendix I and II). A comparison of these amino acid sequences has revealed that, in general, bacterial DHFRs contain about 160 residues while animal DHFRs have approximately 186 amino acids. An additional observation is that animal DHFRs show much greater similarity to each other than do the bacterial enzymes. In animals, about 125 of a total of 186 amino acids of the enzyme are identical. Bacterial DHFRs, on the other hand, are not as homogeneous as animal enzymes, corresponding in only 30 of the approximate 160 residues present in the entire polypeptide. A common feature of DHFRs from both prokaryotes and eukaryotes is a greater homology towards their amino termini compared to their carboxy terminal regions. Another common feature of all DHFR proteins studied to date is a high degree of conservation of the amino acid residues thought to be involved in the binding of MTX and NADPH to the enzyme (see Fig.; Volz et al.,1982).

In contrast to this wealth of information about a number of DHFR proteins from a variety of organisms, very little is known about the yeast enzyme. In fact, so little is known that even the molecular weight of the yeast DHFR protein is in dispute. Various values have been reported including 26,000 (Wu et al., 1976; abstract), 29,000 (C.Geoff, personal communication) and 39,000 Daltons (Blakley, 1984). The 633 bp coding region of the DFR1 gene determined in this study corresponds to a polypeptide of 211 amino acids (Fig. 4 and Fig. 6), with a predicted molecular weight of 24,229.8 Daltons, in general agreement with the report of Wu et al. (1976). The yeast DHFR shows greater homology

towards other DHFR protains in the amino terminal region than in the carboxy terminal residues (Fig. 6). Based on the alignments shown in Fig. 6, the yeast DHFR exhibits approximately 25% sequence homology with the E.coli DHFR, about 20% with both the L.casei and S. faecium proteins and approximately 30% similarity with the animal enzymes.

Of 17 residues known to be involved in MTX binding to the L.casei DHFR (Bolin et al., 1982; Blakley, 1984), 10 are identical in yeast (namely, Ala13, Leu27, Trp29, Phe38, Arg39, Ser63, Phe65, Leu71, Arg74 and Thr141, see Fig. 6). Of these residues, 6 are invariant in all DHFRs analyzed (Ala13, Trp29, Phe38, Leu71, Arg74 and Thr141). Asp at position 34 is found to be conserveed in all animal DHFRs but replaced by Glu in all bacterial DHFRs. The yeast DHFR is similar to other eukaryotes in this respect.

Of 27 residues implicated in the binding of NADPH to the L.casei DHFR (Filman et al., 1982; Blakley, 1984), 13 are identical in the yeast enzyme (viz. Ala13, Ile21, Gly22, Gly25, Leu27, Trp29, Gly57, Arg58, Thr60, Ser63, Gly123, Gly124 and Thr151; see Fig. 6). 8 of these residues are found to be invariant in all DHFRs examined to date (i.e. Ala13, Ile21, Gly22, Trp29, Gly57, Thr60, Gly123 and Gly124). The three others (Gly22, Leu27 and Ser63) appear to be common to all animal DHFRS and some bacterial DHFRs whereas the two remaining residues (Arg58 and Thr60) are conserved in most enzymes, for which sequencing data is available.

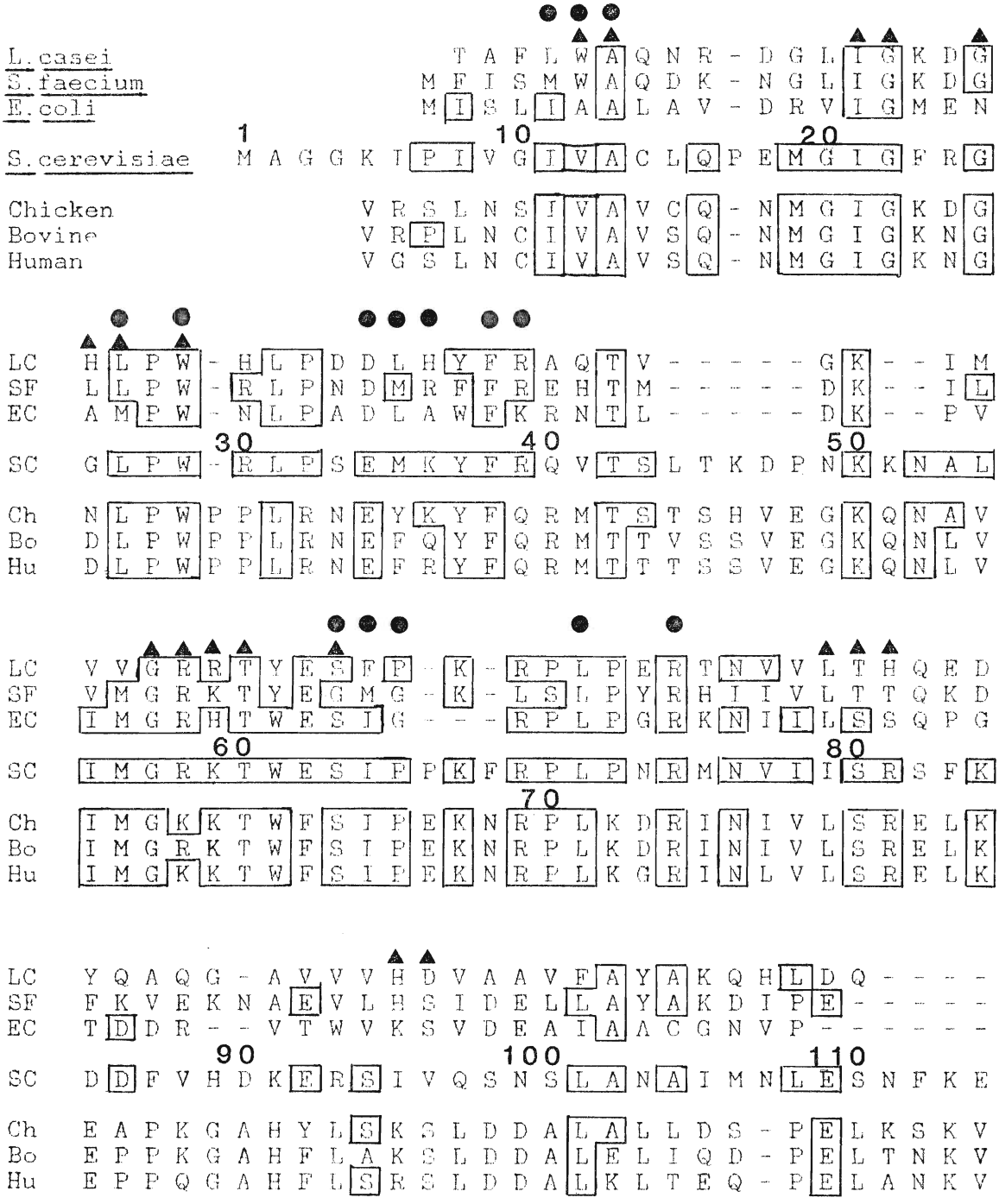
The yeast DHFR is about 25 and 51 residues longer than

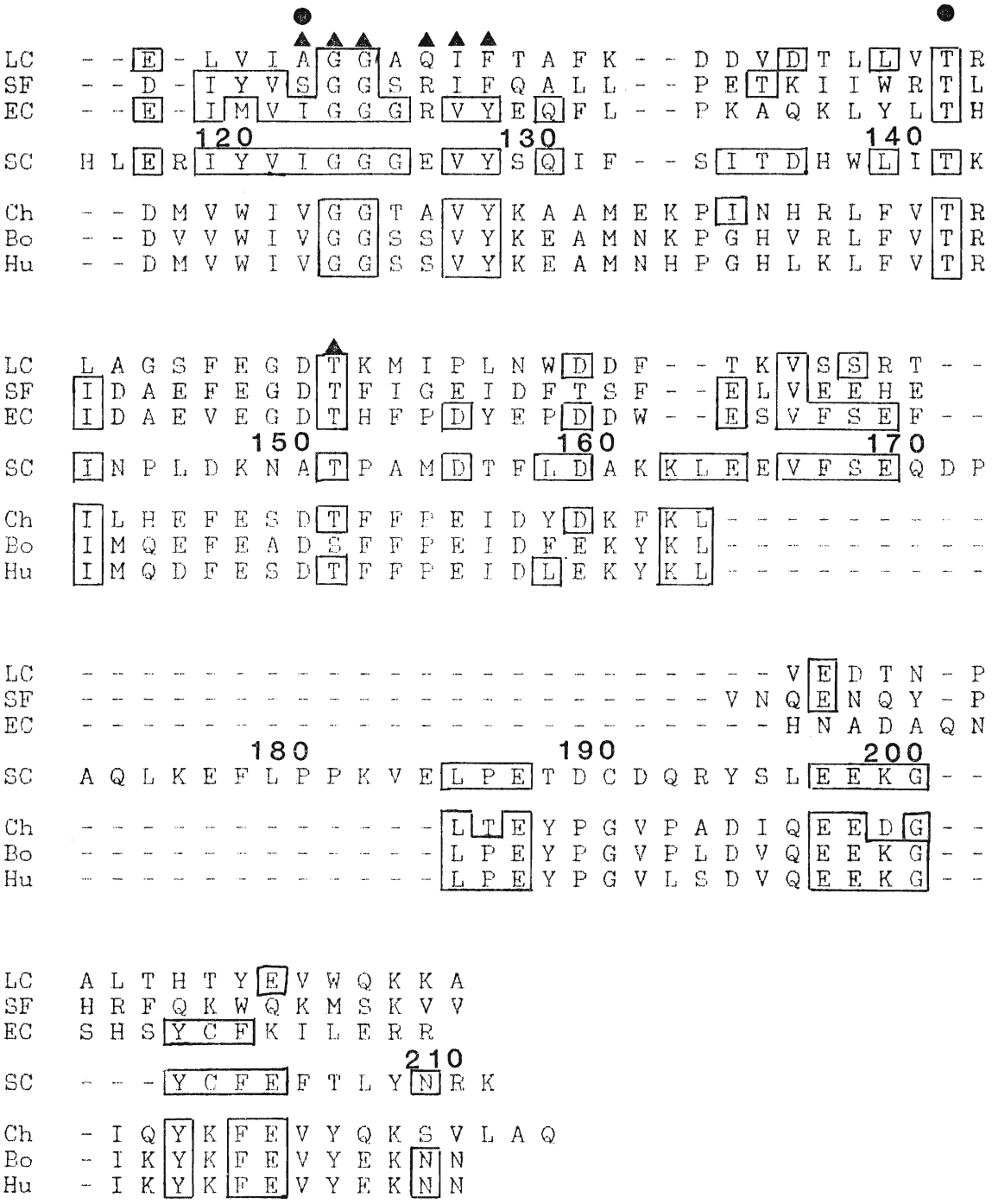
Figure 6. Comparison of the predicted amino acid sequence with other DHFRs

Sources of other DHFR sequences are indicated in Appendix I and II. The numbering system is for the yeast protein. The assignment of the sequences is essentially adapted from Volz et al. (1982). The one letter code for amino acids is as follows: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylanine; G, glycine; H, hisdine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W; tryptophan; Y, tyrosine. Consensus sequences are boxed.

▲: NADPH binding
●: MTX binding

LC: L.casei
SF: S.faecium
EC: E.coli
SC: S.cerevisiae
Ch: Chicken
Bo: Bovine
Hu: Human


L.casei
S.faecium
E.coli
S.cerevisiae
Chicken
Bovine
Human
1
10
20
30
40
50
60
70
80
90
100
110
LC
SF
EC
SC
Ch
Bo
Hu


LC - - E - L V I A G G A Q I F T A F K - - D D V D T L L V T R
SF - - D - I Y V S G G S R I F Q A L L - - P E T K I I W R T L
EC - - E - I M V I G G G R V Y E Q F L - - P K A Q K L Y L T H
120 130 140
SC H L E R I Y V I G G G E V Y S Q I F - - S I T D H W L I T K
Ch - - D M V W I V G G T A V Y K A A M E K P I N H R L F V T R
Bo - - D V V W I V G G S S V Y K E A M N K P G H V R L F V T R
Hu - - D M V W I V G G S S V Y K E A M N H P G H L K L F V T R
LC L A G S F E G D T K M I P L N W D D F - - T K V S S R T - -
SF I D A E F E G D T F I G E I D F T S F - - E L V E E H E
EC I D A E V E G D T H F P D Y E P D D W - - E S V F S E F - -
150 160 170
SC I N P L D K N A T P A M D T F L D A K K L E E V F S E Q D P
Ch I L H E F E S D T F F P E I D Y D K F K L - - - - - - - - -
Bo I M Q E F E A D S F F P E I D F E K Y K L - - - - - - - - -
Hu I M Q D F E S D T F F P E I D L E K Y K L - - - - - - - - -
LC - V E D T N - P
SF - V N Q E N Q Y - P
EC - H N A D A Q N
180 190 200
SC A Q L K E F L P P K V E L P E T D C D Q R Y S L E E K G - -
Ch - - - - - - - - - - - - L T E Y P G V P A D I Q E E D G - -
Bo - - - - - - - - - - - - L P E Y P G V P L D V Q E E K G - -
Hu - - - - - - - - - - - - L P E Y P G V L S D V Q E E K G - -
LC A L T H T Y E V W Q K K A
SF H R F Q K W Q K M S K V V
EC S H S Y C F K I L E R R
210
SC - - - Y C F E F T L Y N R K
Ch - I Q Y K F E V Y Q K S V L A Q
Bo - I K Y K F E V Y E K N N
Hu - I K Y K F E V Y E K N N

animal and bacterial enzymes, respectively. As shown in Fig. 6, when the yeast DHFR is compared with animal DHFRs, apart from 4 amino acid residues that lengthen the amino terminus, the 21 additional residues (in excess of the 186 found in other eukaryotic DHFRs) are located in the region from amino acid 164 to 184. It is noteworthy that this region in animal DHFRs has been defined as a major insertion when compared with bacterial sequences. This particular insertion is quite different than others in eukaryotic proteins in that it does not occur in the loops connecting elements of secondary structure of the polypeptide (Volz et al.,1982; Blakley, 1984). The length of this region in the yeast DHFR is even longer than that of the animal enzymes. The significance of this insertion is unclear at the present time, although it has been suggested that it may contribute to the small but critical changes in the relative positions of elements of secondary structure (Blakley, 1984).

Coincidentally, intron V of the human DHFR gene corresponds to the 3' end of this insertion present in the yeast DHFR gene. Thus, conjunction of exons V and VI of the human DHFR gene is within the codon GA'A coding for the Glu residue at position 187 in the polypeptide while the 3' end of the insertion of the yeast DHFR gene ends at the codon GAG' coding for a glutamate at position 184. These observations prompted a search for any signals which might be involved in RNA splicing in this region. The surprising finding is that the sequence "GTATTT" corresponding to the Val166 and Phe167 residues and sequence "(G)AG" coding for the Glu184 residue are located at the 5'

and 3' end of the insertion, respectively. These sequences greatly resemble the sequences "GTATGT" and "AG" which are the 5' and 3' splice junction consensus sequences of introns of at least 15 yeast nuclear genes (Langford and Gallwitz, 1983; Langford et al., 1984; Teem et al., 1984). In addition to the splice junction conserved sequences within introns, the core sequence "TACTAAC" has been shown to be absolutely required for processing of yeast hnRNAs (Pikielny et al., 1983; Langford et al., 1984). Since this sequence is not found in the insertion sequence of the yeast DFR1 gene, Thouggh, it appears that there are sequences present in this region which are necessary but not sufficient for nuclear intron splicing.

The search for intron consensus homology with the yeast insertion sequence aslo revealed a partial homology with the consensus sequences of class II mitocondrial introns (Keller and Michel, 1985), as shown below:

```
DFR1:      5'...GAGC...U...GAAA.....................CUA...A.3'
classII:   5'...GAGC...U...GAAA...A.G....GTT....G...CUC...A.3'
```

The significance, if any, of these homologies with intron consensus sequences of both nuclear and mitochondrial genes remains unclear.

II. Construction of DFR1:lacZ Gene Fusions

As mentioned previously (see Introduction), lacZ fusions have been successfully employed in a variety of genetic and biochemical studies in yeast. lacZ fusions have been particularly valuable in studies of the regulation of gene

expression. Thus, it was thought that construction of a DFR1:lacZ fusion would greatly facilitate the study of the regulation of DFR1 gene expression in yeast. Previously in our laboratory, an attempt was made to construct such fusions by an in vivo selection approach which was based on spontaneous or induced nonhomologous recombination of the regulatory sequence of the DFR1 with the continuous lacZ gene on the plasmid (D. Steele, Undergraduate Thesis, Brock Universoty). This study was hampered by lack of information about the physcial organization of the cloned DFR1 gene. With the availibility of the DNA sequence data obtained in this study, another approach became practicable. This method was based on the deletions in vitro of the 3' terminus of the coding region of the DFR1 with double-stranded DNA exonuclease Bal31, followed by the selection in vivo of the functional fusion constructions with the lacZ gene (Guarente, 1983; Rose and Botstein, 1983a). This in vitro method possesses many advantages. For example, the deletion end points of the DFR1 fragment could be chosen to lie far from or close to the start codon of the DFR1 DNA fragment to be fused to the lacZ.

In order to construct a hybrid gene between the yeast DFR1 and the E.coli lacZ gene, plasmid pLG669-Z was employed. In addition to standard features of an E.coli-yeast shuttle vector, this plasmid had a 'lacZ fragment in which the 5' end of the lacZ coding sequence including the ATG codon had been deleted. The 5' end point of this fragment was immediately preceded by a unique BamH1 restriction site. Since there was no

BamH1 restriction site within the coding region of the DFR1 gene, the requisite BamH1 site in the coding region was then introduced by the attachment of synthetic oligonucleotide BamH1 linker onto the ends of Bal31-generated DFR1' fragments. To produce the DFR1:lacZ fusions, another unique restriction site Sal1, upstream from the BamH1 site in the plasmid pLG669-Z, was used to orientate the insertion of the DFR1' fragments, in which the appropriate 3' coding sequences had been deleted. Due to the consideration that the polypepide of the DHFR protein might have some particular function on the regulation of its own expression, it was decided to construct two sets of DFR1:lacZ fusions which retained either almost the entire coding region of the DFR1 gene (pEFZ fusions) or only a small number of the amino terminal codons of the DFR1 gene (pHFZ fusions). It was thought that these two sets of fusions might have different regulatory properties.

The procedure used in construction of the pEFZ fusions is shown in Fig.7. By this approach, a total of 11 E. coli transformants with independent pEFZ fusion plasmids were obtained. Examination of these transformants on selective M9 medium containing Tm (2ug per ml) indicated that all of them failed to confer Tm resistance upon the bacterial host, indicating the loss of the DFR1 function. Restriction mapping of some of these pEFZ fusion plasmids are present in Fig. 8. Digestion with restriction enzyme BamH1 and Sal1 resulted in the expected bands of about 1.4 kb (Fig. 8A), which indicated the correct insertion of the Bal31-generated DFR1' fragment into

Figure 7. Construction of pEFZ fusion plasmids

The experimental procedure was described in "Methods". Abbreviations for restriction sites are as folowing: B, BamH1; E, EcoR1; H, HindIII; P, Pst1; Pv, PvuII; S, Sal1; X, XhoII.

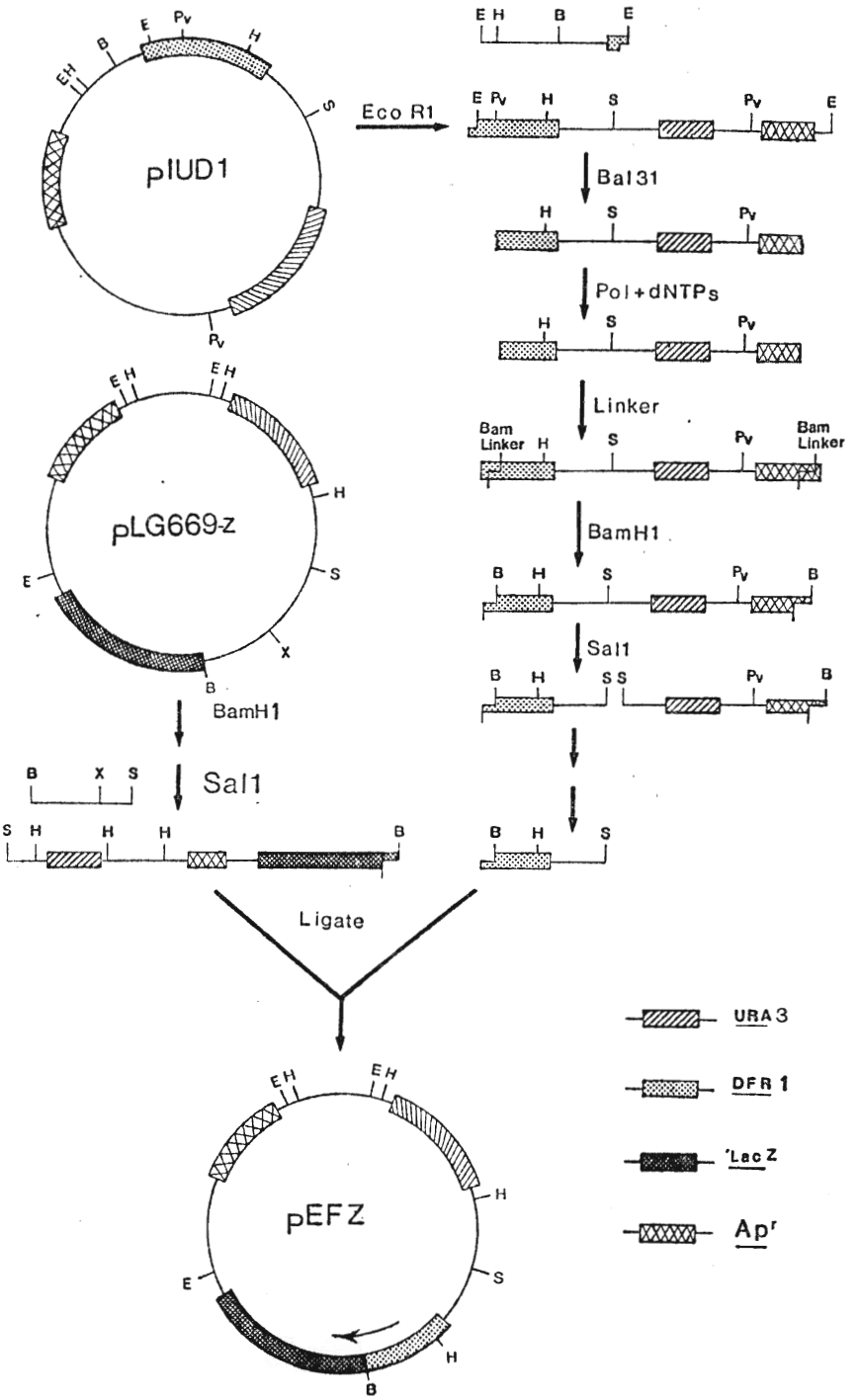

pIUD1
Eco R1
Bal31
Pol+dNTPs
Linker
BamH1
Sal1
pLG669-z
BamH1
Sal1
Ligate
pEFZ
URA 3
DFR 1
'Lac Z
Ap^r

Figure 8. Restriction mapping analysis of the fusion plasmids

Plasmid DNA samples were prepared by the rapid mini-procedure (see Methods). After digested with appropriate restriction enzymes, DNA fragments were separated by 0.7% agarose gel electrophoresis. Lambda DNA digested with restriction enzyme HindIII was used as size marker (23, 9.4, 6.7, 2.3, 2.0, and 0.4 kb).

(A) : pEFZ plasmids were digested with BamH1 and Sal1
(B) : pEFZ plasmids were digested with HindIII.

lane 1: pEFZ-2.
lane 2: pEFZ-3.
lane 3: pEFZ-4.
lane 4: pEFZ-8.
lane 5: pEFZ-9.
lane 6: pEFZ-10.
lane 7: pEFZ-11.
lane 8: lambda DNA digested with HindIII

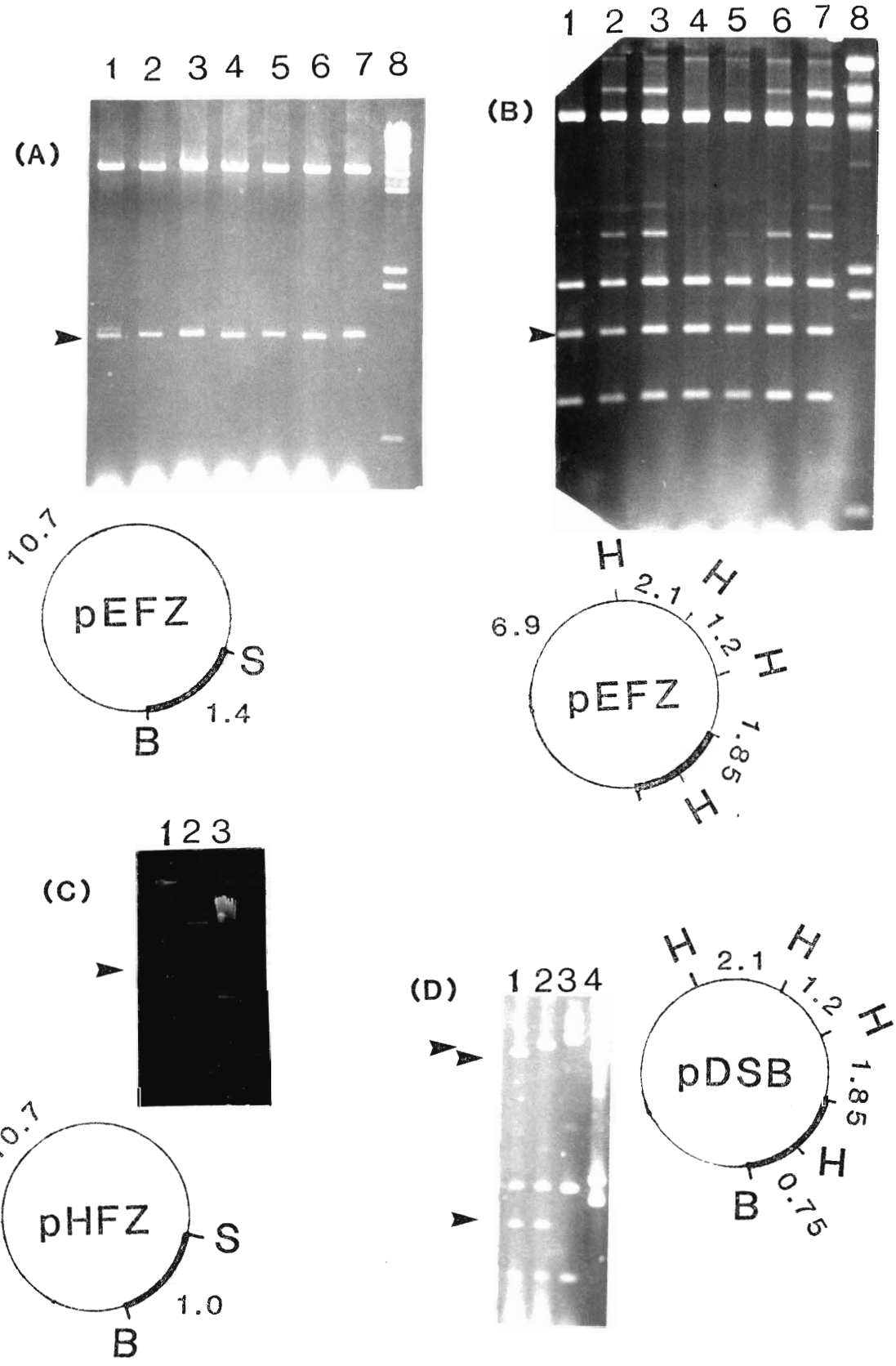
(c) :

lane 1: uncut pHFZ-12 plasmid DNA.
lane 2: pHFZ-12 digested with BamH1 and Sal1.
lane 3: lambda DNA digested with HindIII.

(D) :

lane 1: pDSB1-Z digested with BamH1 and HindIII.
lane 2: pDSB1-Z digested with HindIII.
lane 3: pLG669-Z digested with HindIII.
lane 4: lambda DNA digested with HindIII.

▬▬: DFR1 DNA.
——: pLG669-Z vector DNA.


1 2 3 4 5 6 7 8
(A)
1 2 3 4 5 6 7 8
(B)
10.7
pEFZ
S
1.4
B
H
2.1
H
1.2
6.9
H
pEFZ
1.85
H
1 2 3
(C)
10.7
pHFZ
S
1.0
B
(D)
1 2 3 4
H
2.1
H
1.2
H
1.85
pDSB
H
0.75
B

the vector pLG669-Z. Digestion with restriction enzyme HindIII further confirmed this by revealing the existence of the HindIII restriction site within the coding region of the DFR1 gene (Fig. 8B).

The pHFZ fusion plasmids were constructed according to the procedure shown in Fig. 9. Several hundred bacterial transformants with independent pHFZ fusion plasmids were obtained. 18 independent transformants examined all showed sensitivity to Tm (2ug/ml). The restriction mapping analysis of the plasmid pHFZ-12 is shown in Fig. 8C. The plasmid pDSB1-Z was constructed, for use as a control, by an insertion of the intact cloned Sal1/BamH1 yeast genomic fragment into the vector pLG669-Z, so that the intact DFR1 gene was laid upstream of the 'lacZ fragment. As shown in Fig. 2, a E. coli host of this construction was resistant to the growth inhibition of Tm.

III. Expression of DFR1:lacZ Gene Fusions In E. coli And S. cerevisiae

Expression of hybrid beta-gal activity of fusion products derived from the DFR1:lacZ constructions was measured qualititatively on agar solid plate. Functional expression of the fused genes was evidenced by the appearance pf blue colonies growing on selection plates containing chromogenic substrate X-gal. Bacterial transformants with 18 independent pHFZ fusion plasmids all exhibited blue colour when grown on X-gal-L selection agar medium after one-day incubation (Fig.11B). In contrast to these pHFZ fusions, transformants with 11 independent

Figure 9. Construction of pHFZ fusion plasmids

The experimental procedure was described in "Methods". Abbreviations for restriction sites are the same as in the Fig. 7.

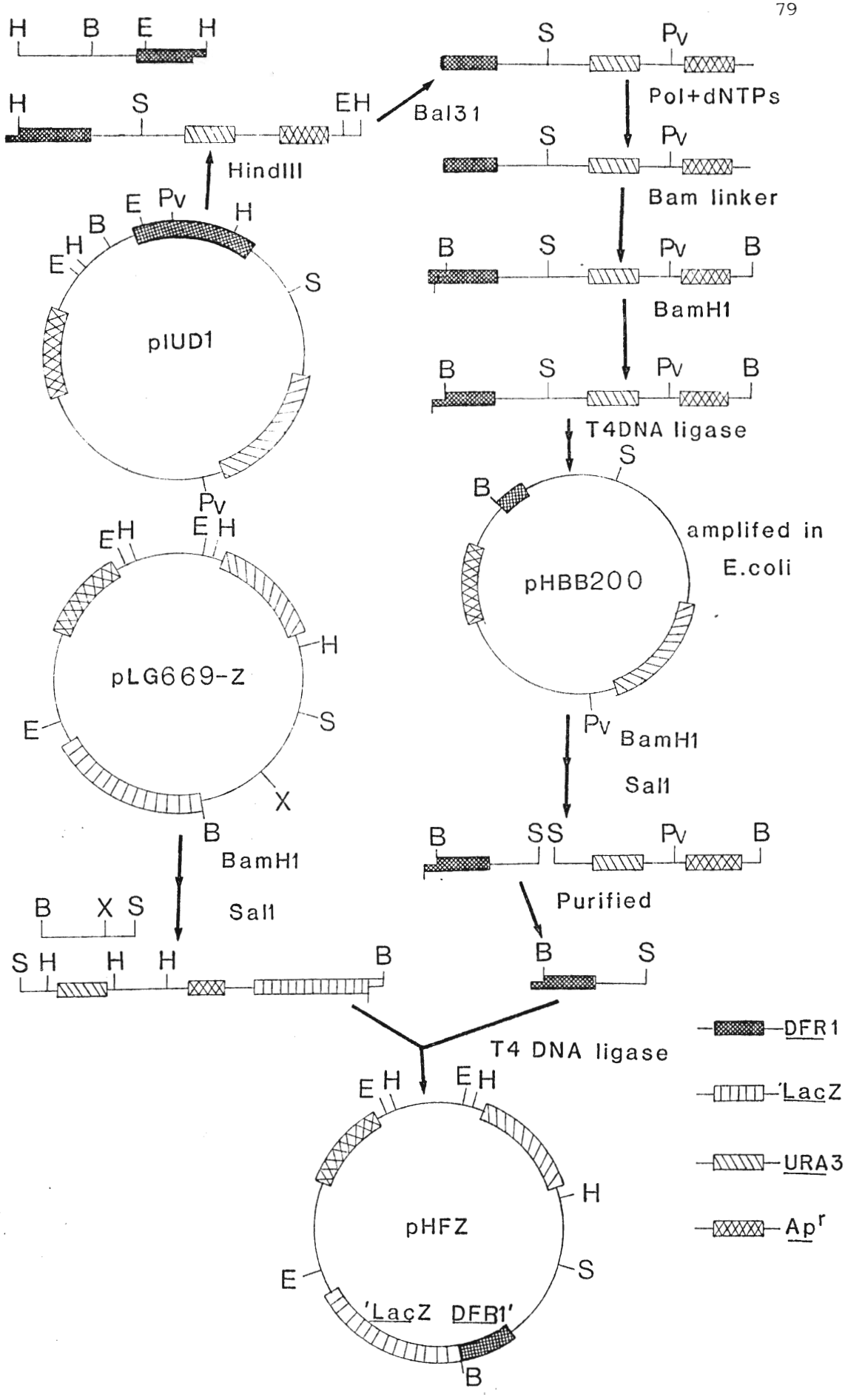
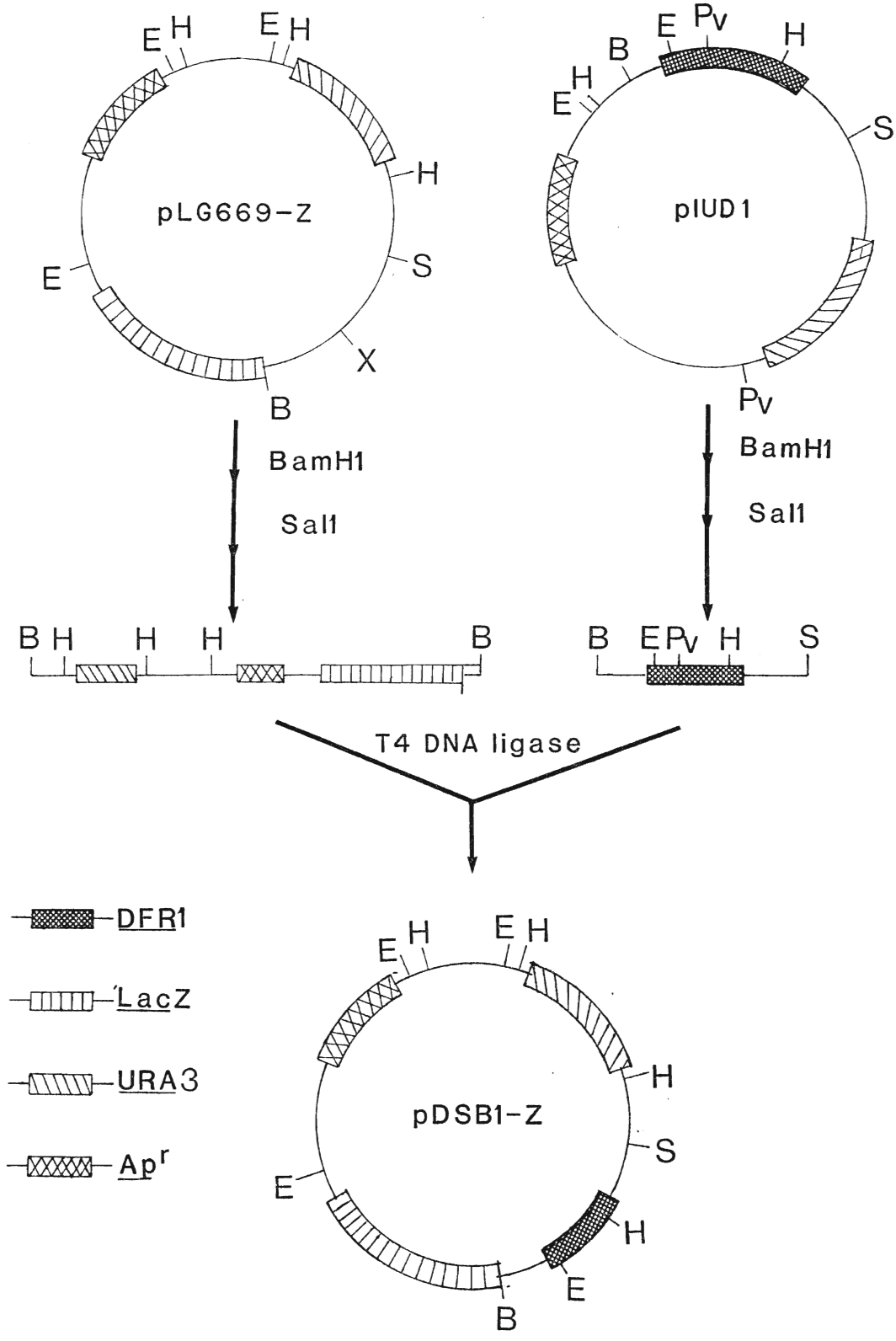

H B E H
H S EH
HindIII
Bal31
S Pv
Pol+dNTPs
Bam linker
BamH1
T4DNA ligase
pIUD1
pHBB200
amplifed in
E.coli
pLG669-Z
BamH1
Sal1
Purified
T4 DNA ligase
pHFZ
'LacZ DFR1'
DFR1
'LacZ
URA3
Ap^r

Figure 10. Construction of plasmid pDSB1-Z

Experimental procedure is described in "Methods".


pLG669-Z
pIUD1
BamH1
Sal1
BamH1
Sal1
T4 DNA ligase
pDSB1-Z
DFR1
LacZ
URA3
Ap^r

pEFZ fusion plasmids showed no blue colour even after prolonged incubation for periods up to one week (Fig. 11A). A similar observation was obtained for transformant with the control plasmid pDSB1-Z (Fig. 11B) Above observations indicated that the pHFZ fusions directed higher levels of beta-galactosidase than did the pEFZ fusions in E.coli.

Detection of beta-gal activity in yeast was carried out by using buffered SD medium (pH 7.0). In contrast to the results observed in bacteria, the expression pattern of the pHFZ and pEFZ fusion plasmids in yeast exhibited obvious two classes. Of the 11 independent pEFZ fusions examined, only 6 directed beta-galactosidase synthesis (ie. pEFZ-2, pEFZ-4, pEFZ-5, pEFZ-8, pEFZ-9 and pEFZ-11; see Fig. 12A). Similarly, among 18 pHFZ fusions, only 7 expressed beta-galactosidase activity (namely, pHFZ-2, pHFZ-4, pHFZ-6, pHFZ-8, pHFZ-10, pHFZ-12 and pHFZ-16; see Fig. 12B). Visible blue colour in the yeast colonies was developed only by increasing the concentration of X-gal in the buffered selective medium up to 200 ug per ml and prolonging the incubation for at least 15 days. This observation indicated that the levels of the beta-galactosidase activity derived from the pHFZ and pEFZ fusion plasmids in yeast cells were relatively low. This was confirmed by one other functional CYC1:lac fusion carried by plasmid pLG669-Z which exhibited strong blue colour after even only one day incubation, at the standard assay condition. However, the control plasmid pDSB1-Z showed no beta-galactosidase activity.

For more precise measurement of levels of beta-galactosidase

Figure 11. Expression of DFR1:lacZ fusions in E.coli

Exponential cells (MC1064/pEFZ or pHFZ) from a overnight culture grown in AMP-LB medium were spotted on the same selective plate supplemented with 200 ug X-gal per ml. Shown is the observation that the plates had been incubated at 37°C for two days.

A.
1: pEFZ-2
2 pEFZ-4
3: pEFZ-5
4: pEFZ-6
5: pEFZ-8
6: pEFZ-9
7: pEFZ-11
8: pDSB1-Z

B.
1: pHFZ-1
2: pHFZ-2
3: pHFZ-3
4: pHFZ-4
5: pHFZ-5
6: pHFZ-6
7: pHFZ-7
8: pHFZ-8
9: pHFZ-9
10: pHFZ-10
11: pHFZ-11
12: pHFZ-12
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17: pHFZ-17
18: pHFZ-18

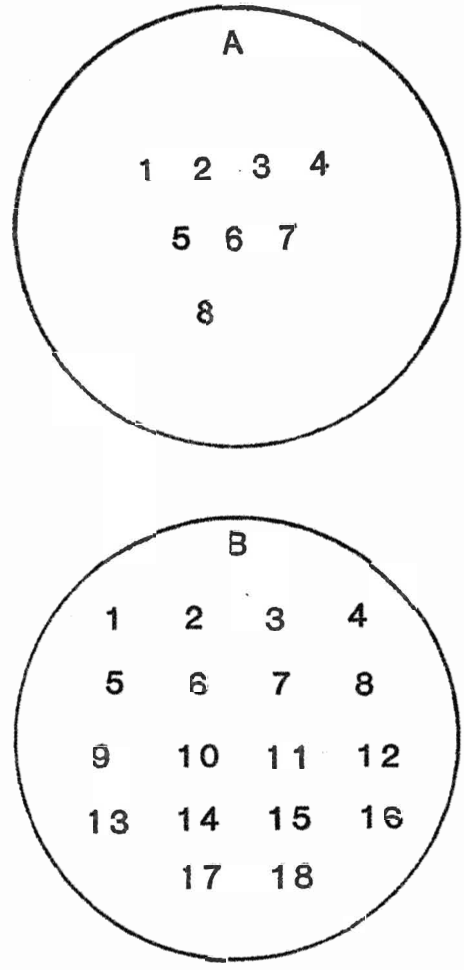
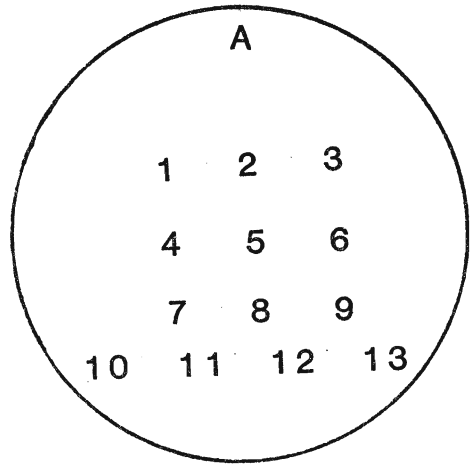


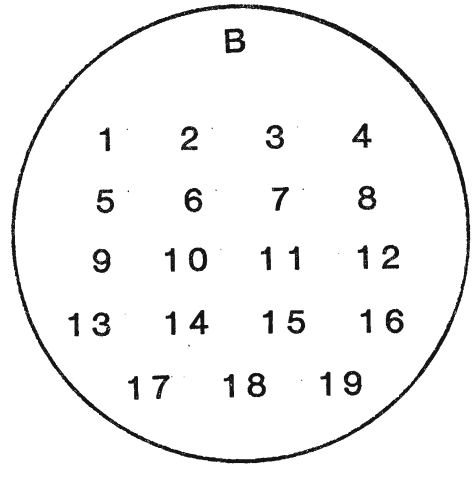
Figure 12. Expression of the DFR1:lacZ fusions in yeast

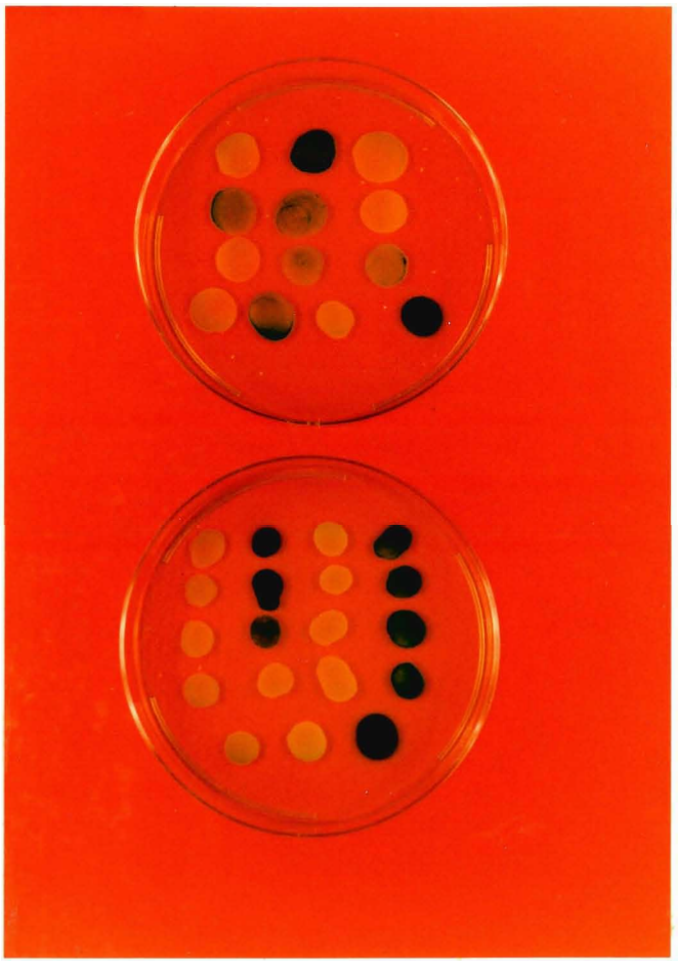
Yeast cells containing the plasmids, previously grown in SD selection medium, were spotted on buffered SD selection medium supplemented with 200ug X-gal per ml. The results showed here were the observations after incubation at 30°C for 20 days.

A.
1: pEFZ-1
2: pEFZ-2
3: pEFZ-3
4: pEFZ-4
5: pEFZ-5
6: pEFZ-6
7: pEFZ-7
8: pEFZ-8
9: pEFZ-9
10: pEFZ-10
11: pEFZ-11
12: pDSB1-Z
13: pLG669-Z



B.
1: pHFZ-1
2: pHFZ-2
3: pHFZ-3
4: pHFZ-4
5: pHFZ-5
6: pHFZ-6
7: pHFZ-7
8: pHFZ-8
9: pHFZ-9
10: pHFZ-10
11: pHFZ-11
12: pHFZ-12
13: pHFZ-13
14: pHFZ-14
15: pHFZ-15
16: pHFZ-16
17: pHFZ-17
18: pHFZ-18
19: pLG669-Z





activity, quantitive assays were performed by the permeabilized cell method or the cell lysis method. As shown in Table 5, no beta-galactosidase activity could be detected in bacterial transformants with pEFZ fusion plasmids. In contrast to these construction, pHFZ fusion plasmids all directed relatively much higher levels of beta-galactosidase activity in E.coli (Table 5). These results were consistent with those observed in the plate assays. However, if the assay reaction was allowed to last for up to 24 hs, the extremely low levels of the enzyme activity, derived from all pEFZ fusions and the pDSB1-Z plasmid as well, could be detected by this cell lysis method. In yeast, consistent with the observations in plate assay, 11 of 18 pHFZ fusion plasmids which expressed beta-galactosidase in E.coli failed to exhibited the enzyme activity in yeast (pHFZ-1, pHFZ-3, pHFZ-5, pHFZ-7, pHFZ9, pHFZ-11, pHFZ-13, pHFZ-14, pHFG-15, pHFG-17 and pHFZ-18; see Table 5), while the 7 others (pHFZ-2, pHFZ-4, pHFZ-6, pHFZ-8, pHFZ-10, pHFZ-12 and pHFZ-16; see Table 5) expressed relatively higher levels of enzyme activity. Similarly, among the 11 pEFZ fusions, 6 (pEFZ-2, pEFZ-4, pEFZ-5, pEFZ-8, pEFZ-9 and pEFZ-11) expressed higher levels of beta-gal activity (Table 5), whereas the 5 others had no the enzyme activity or extremely low activity (pEFZ-1, pEFZ-6, pEFZ-7 and pEFZ-10; Table 5). In general, the levels of enzyme activity derived from pHFZ fusions were higher than those derived from pEF fusions. The same expression patterns of these fusion plasmids were observed by both the cell lysis method and the permeabilized cell method.

Table 5. Expression of DFR1:lacZ fusions

Fusion plasmid		β-Galactosidase activity E.coli	Yeast
Host	(M1/2B)	0	0
pLG669-Z	(CYC1:lacZ)	-	5.496
pDSB1-Z	(DFR1, lacZ)	0	0
pEFZ-1	(DFR1:lacZ)	-	0.002
pEFZ-2		0	0.654
pEFZ-3		-	0.000
PEFZ-4		0	0.601
pEFZ-5		0	0.658
pEFZ-6		0	0.002
pEFZ-7		-	0.005
pEFZ-8		0	0.670
pEFZ-9		0	0.536
pEFZ-10		-	0.002
pEFZ-11		0	0.492
pHFZ-1	(DFR1:lacZ)	29	0.000
pHFZ-2		17	1.708
PHFZ-3		7	0.000
pHFZ-4		8	0.784
pHFZ-5		11	0.000
pHFZ-6		40	1.180
pHFZ-7		2	0.000
pHFZ-8		22	1.570
pHFZ-9		12	0.000
pHFZ-10		38	1.622
pHFZ-11		21	0.000
pHFZ-12		15	1.138
pHFZ-13		8	0.000
pHFZ-14		11	0.000
pHFZ-15		23	0.000
pHFZ-16		40	1.070
pHFZ-17		8	0.000
pHFZ-18		7	0.000

-: not tested. Assay of the enzyme activity in yeast cells was performed by the permeabilized cell method as described in Methods. Bacterial assay was the standard (Miller, 1972).

It is likely that the fusions which expressed higher level of beta-gal activity in yeast cells represent those in which the two DNA fragments were joined in frame, thereby resulting in the functional expression of the hybrid gene in yeast. Such a conclusion could be easily confirmed by DNA sequence analysis of the fusion junctions through a simple cloning scheme of these fragments in M13. This would be greatly aided by availability of the BamH1 restriction site in the junctions and the DNA sequence of the DFR1 gene determined in this study. The isolation of functional DFR1:LacZ fusions should provide very useful tools for studies of the expression and regulation of the yeast DFR1 gene.

IV. UV-induced Expression of a DFR1:lacZ Fusion in Yeast

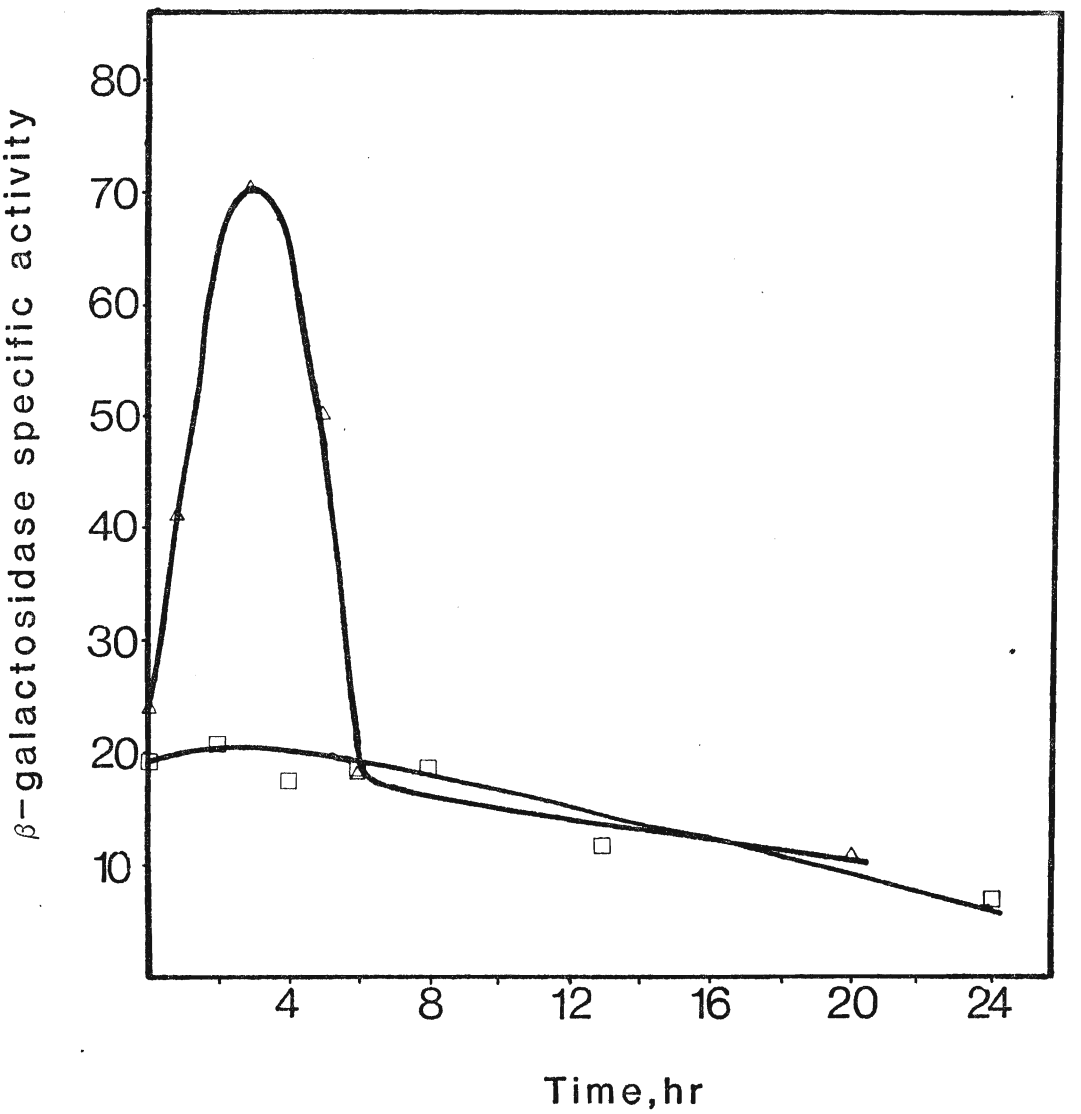
As mentioned previously (see Introduction), intracellular levels of DHFR activity are responsive to a variety of modulators such as serum, antifolate and virus. Recently, several studies have demonstrated that amplification of DHFR gene in mammalian cells is enhanced by pretreatment with a variety of chemical and physical carcinogens such as hydroxyurea or UV (Brown et al., 1983;; Tlsty et al., 1984; Johnston, et al., 1986; Kleinberger et al., 1986).

Preliminary studies with a DFR1:lacZ fusion plasmid (pHFZ-12) in yeast strain M1/2B demonstrated that the beta-gal activities derived from the fusion plasmid were inducible by treatment with UV light (see Fig. 13) As shown, the enzyme activity in the UV-treated cells was increased after the

Figure 13. Kinetics of UV induction of β-galactosidase in strain M1/2B containing a DFR1:lacZ fusion plasmid.

△ : UV treatment
□ : no traetment

Yeast M1/2B cells containing pHFZ-12 were grown in SD medium supplemented with tryptophan to mid-log phase. 5 ml of this culture was irradiated in dishes with covers removed at a fluence of about 1 $J/m^{-2}/s'$ for 30 seconds. 30 ml of thus treated culture was incubated at 30°C. 5 ml samples were collected at each certain time after the treatment, which were used for the assay of beta-galactosidase activity by the permeabilized cell method. Non-treated culture was used as a control.


β-galactosidase specific activity
80
70
60
50
40
30
20
10
4
8
12
16
20
24
Time, hr

treatment and reached the highest level after subsequent growth for about 3 h. This induced level of the enzyme activity declined to the level of that of the non-treated cells after about 6 h growth. In contrast to these observations, the cells not treated with UV light showed a stable decrease of the beta-gal activity during the period that cells were entering stationary phase. The observation made in the experiments described here suggested that expression of the DFR1 was inducible by UV. It is worthy of note that the DFR1:lacZ fusion used in this study was carried on a multicopy plasmid. Thus, it is likely that the induced levels of the enzyme activity are not associated with gene amplication. It is clear that more detailed experiments are required to confirm this and to understand the nature of this UV induction of yeast DHFR expression. However, it is an intriguing finding and, if supported by further experiments, may indicate an novel role of DHFR in the cellular response to DNA damage which has heretofore not been suspected.

V. Construction of a Yeast DFR1 Null Allele by Gene Disruption

In spite of the fact that several laboratories have attempted to isolate a DHFR-deficient strain of S .cerevisiae by classical techniques of mutant isolation (J.G. Little, personal communication), to my knowledge, no such strain is yet available. The absence of a DHFR-deficient mutant has limited studies of the yeast DHFR function. As such a mutant strain would be extremely useful for studies in this regard. It was hoped that

a yeast DFR1 null allele could be constructed in vivo by gene disruption using the cloned DFR1 gene.

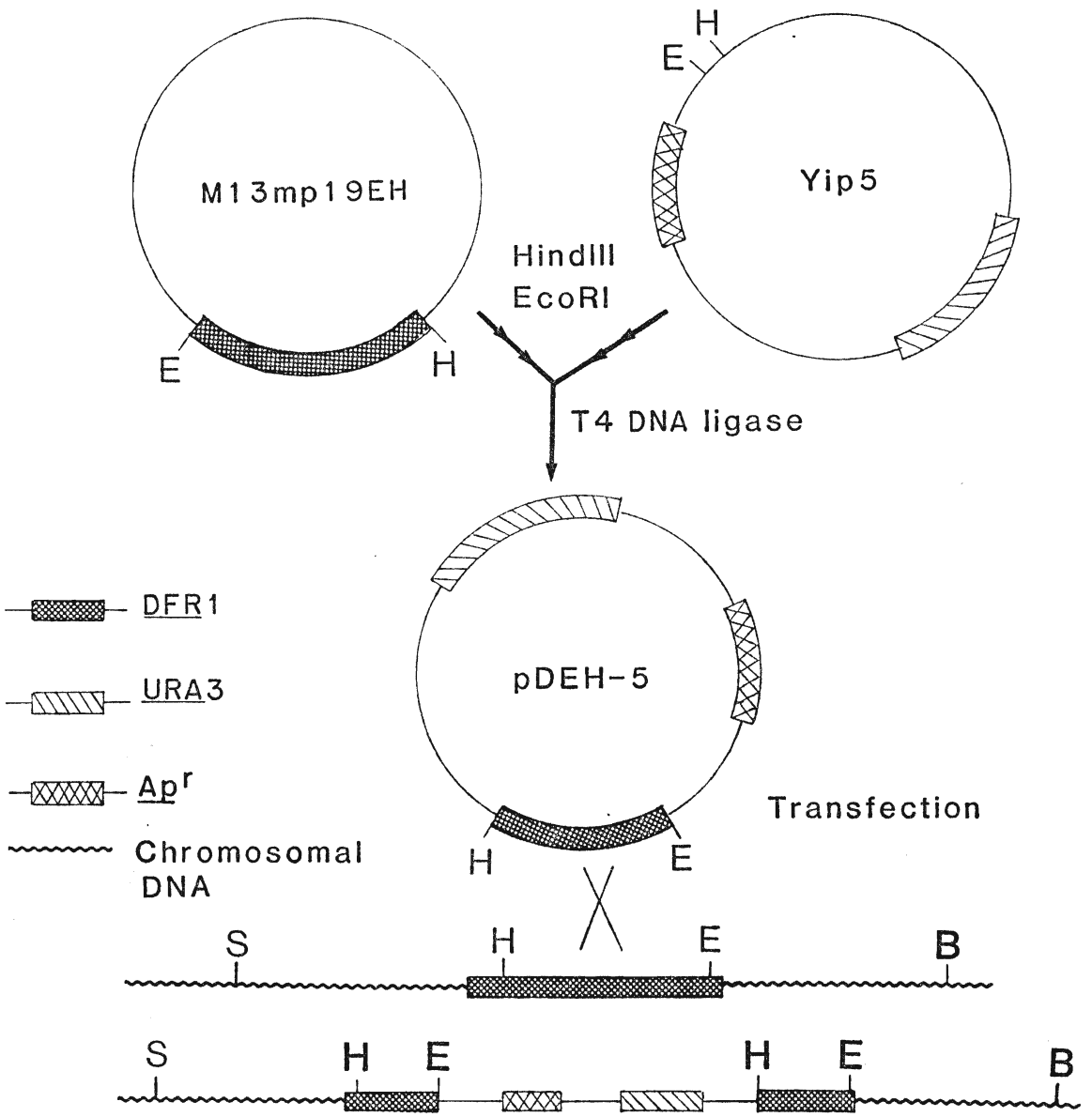
The general experimental approach used in gene disruption studies was that described by Rothstein (1983) and by Shortle et al.(1982). The cloned DFR1 gene was inactivated in vitro by insertion of a functional URA3 gene within the coding region or alternately by deletion of both 5' and 3' flanking sequences. The dfr1 null allele thus constructed in vitro was then integrated into the yeast genome by transfection , thereby disrupting the functional DFR1 chromosomal locus.

Figure 14 outlines the procedure by which a HindIII/EcoR1 fragment which laid entirely within the coding region of the DFR1 was inserted into the integrating vector Yip5. The resulting plasmid (pDEH-5) carried a functional yeast URA3 gene which could be used as a selectable marker in a ura3 host, but it possessed no DFR1 function. Restriction enzyme analysis of this construction is shown in fig. 16.

Integration, by a single homologous recombination event between the chromosomal DFR1 gene and the internal coding sequence in the plasmid would result in a direct repeat of the DFR1 gene sequence in which one fragment contained a deletion in its 5' end and the repeated sequence lacked the 3' region. Thus, each copy of the DFR1 gene in the chromosome would now be incomplete (see Fig. 14). This procedure has the disadvantage that the resulting strain would have direct repeat sequences which would give rise to an excision of the plasmid DNA via homologous recombination at a relatively high frequency, thereby,

Figure 14. Construction of plasmid pDEH-5 and disruption of DFR1 locus

Experimental procedure used in the construction of pDEF-5 is described in "Methods". Also shown is the principle of the disruption of the DFR1 locus by using this construction.


M13mp19EH
E
H
H
E
Yip5
HindIII
EcoRI
T4 DNA ligase
pDEH-5
H
E
Transfection
DFR1
URA3
Ap^r
Chromosomal DNA
S
H
E
B
S
H
E
H
E
B

resulting in a restoration of functional DFR1 locus and loss of the selective marker. However, this plasmid construction could be used to transfect yeast cells in a relatively straightforward procedure without previous digestion of the DNA with restriction enzymes.

For other experiments it is more desirable to employ another procedure by which a more stable dhfr- mutant strain could be constructed. Fig. 15 shows the procedure used in construction of plasmid pNBU-3 which carried a DFR1 null allele by insertion of the yeast URA3 gene into its coding region. Characterization of this DFR1 disruption plasmid with restriction enzymes is present in Fig. 16. In addition, shown in Fig. 17 is the phenotypic characterization of plasmids pNHO-1 and pNBU-3 which failed to transform an E.coli host to Tm resistance and plasmid pNBD-2 containing the DFR1 allele which confered Tm resistance upon the same host. These observations indicated that the function of the DFR1 gene carried by plasmid pNBU-3 had been disrupted by the URA3 sequence inserted into the internal HindIII site.

In order to direct the integration of the modified DNA fragment into the DFR1 locus, plasmid pNBU-3 was digested with restriction enzyme Sal1 and BamH1 prior to transfection. Thus, both ends of the DNA fragment had homologous sequences with those in of chromosomal DFR1 locus. A double crossover in both sides of the URA3 gene would result in an exchange of the non-functional disrupted dfr1 gene for the DNA fragment into the functional DFR1 locus (Fig. 18). Although this construction was

Figure 15. Construction of plasmid pNBU-3

Detailed experimental procedure was described in "Methods". Abbreviations for restriction sites are the same as in Fig. 7.

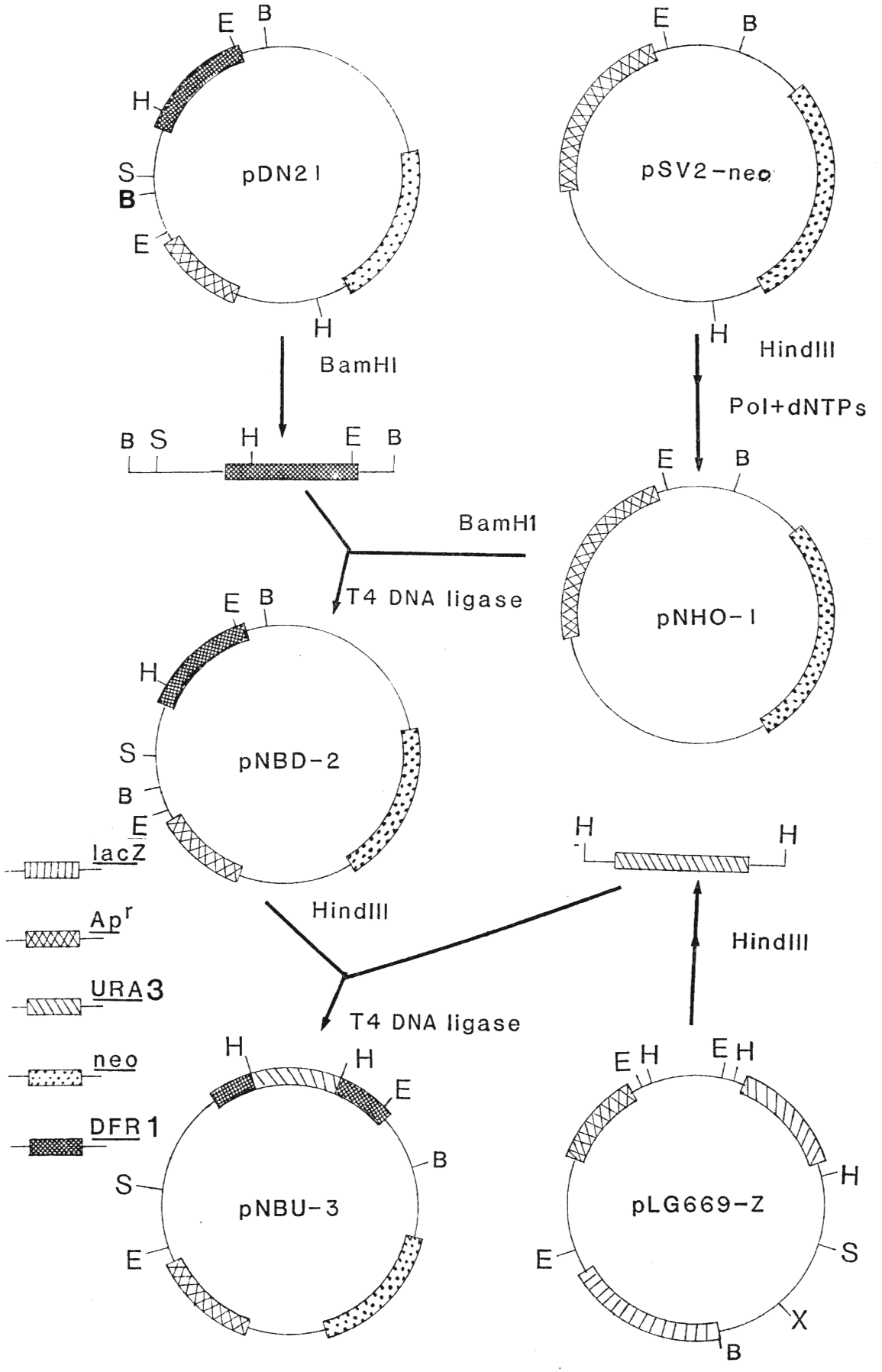

pDN2 I
BamHI
B S H E B
BamH1
T4 DNA ligase
pSV2-neo
HindIII
Pol+dNTPs
pNHO-1
pNBD-2
lacZ
Ap^r
URA3
neo
DFR1
HindIII
H H
HindIII
T4 DNA ligase
pNBU-3
pLG669-Z

Figure 16. Restriction mapping analysis of plasmids pDEH-5 and pNBU-3

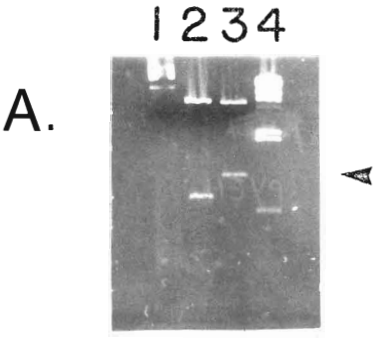
Plasmid DNA samples were preparated by the rapid minu- procedure (see "Methods"). After digested with appropriate restriction enzymes, DNA fragments were separated by 0.7% agarose gel electrophoresis. Lambda DNA digested with restriction enzyme HindIII was used as size marked.

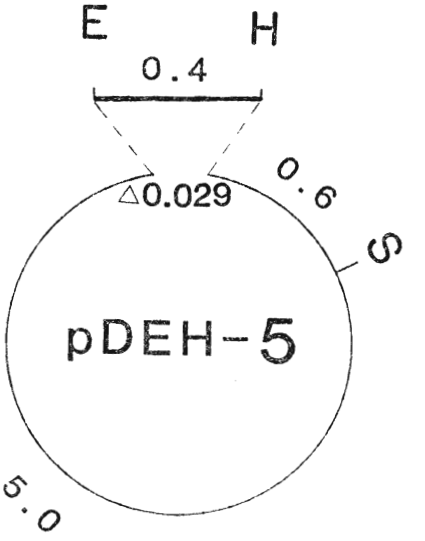
A. Characterization of plasmid pDEH-5.

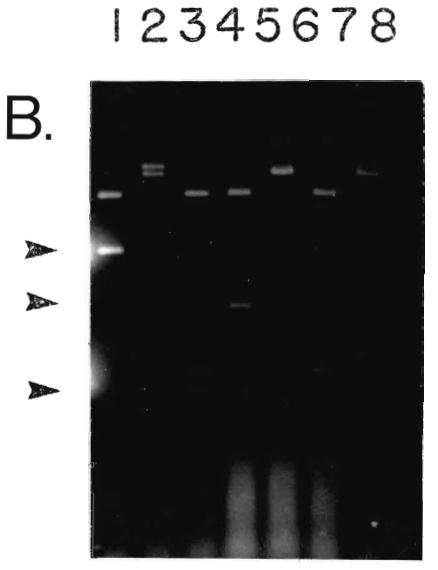
lane 1: pDEH-5 not digested.
lane 2: Yip5 digested with BamH1 and HindIII.
lane 3: pDEH-5 digested with BamH1 and HindIII.
lane 4: lambda DNA digested with HindIII.

B. Characterization of plasmid pBNU-3.

lane 1: pNBU-3 digested with BamH1.
lane 2: pNBU-3 digested with HindIII.
lane 3: pNBU-3 digested with BamH1 and HindIII.
lane 4: pNBD-2 digested with BamH1.
lane 5: pNBD-2 digested with HindIII.
lane 6: pNBD-2 digested with BamH1 and HindIII.
lane 7: pLG669-Z digested with HindIII.
lane 8: lambda DNA digested with HindIII.


1 2 3 4
A.


E
H
0.4
△0.029
0.6
S
pDEH-5
5.0


1 2 3 4 5 6 7 8
B.

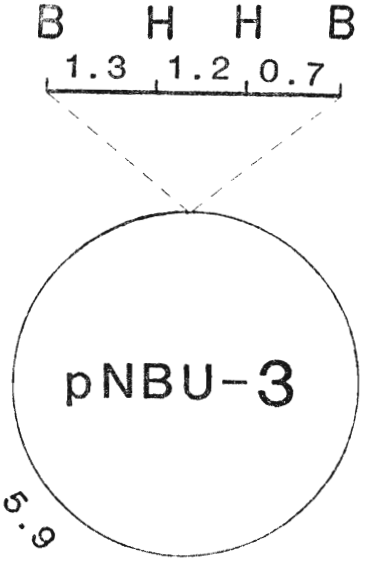

B
H
H
B
1.3
1.2
0.7
pNBU-3
5.9

Figure 17. Phenotypic characterization of plasmids pNHO-1, pNBD-2 and pNBU-3.

○ : JF1754/pNHO-1. (no DFR1)
△ : JF1754/pNBD-2. (DFR1)
□ : JF1754/pNBU-3. (disrupted DFR1)

Exponential cells, previously grown in Amp-LB medium, were harvested by centrifugation and resuspended in M9 medium supplemented with Met, His, Leu and Tm (2ug/ml). Aliquots were taken after appropriate period of incubation at 37°C and the growth of the cultures was determined by their OD values at 600 nm.

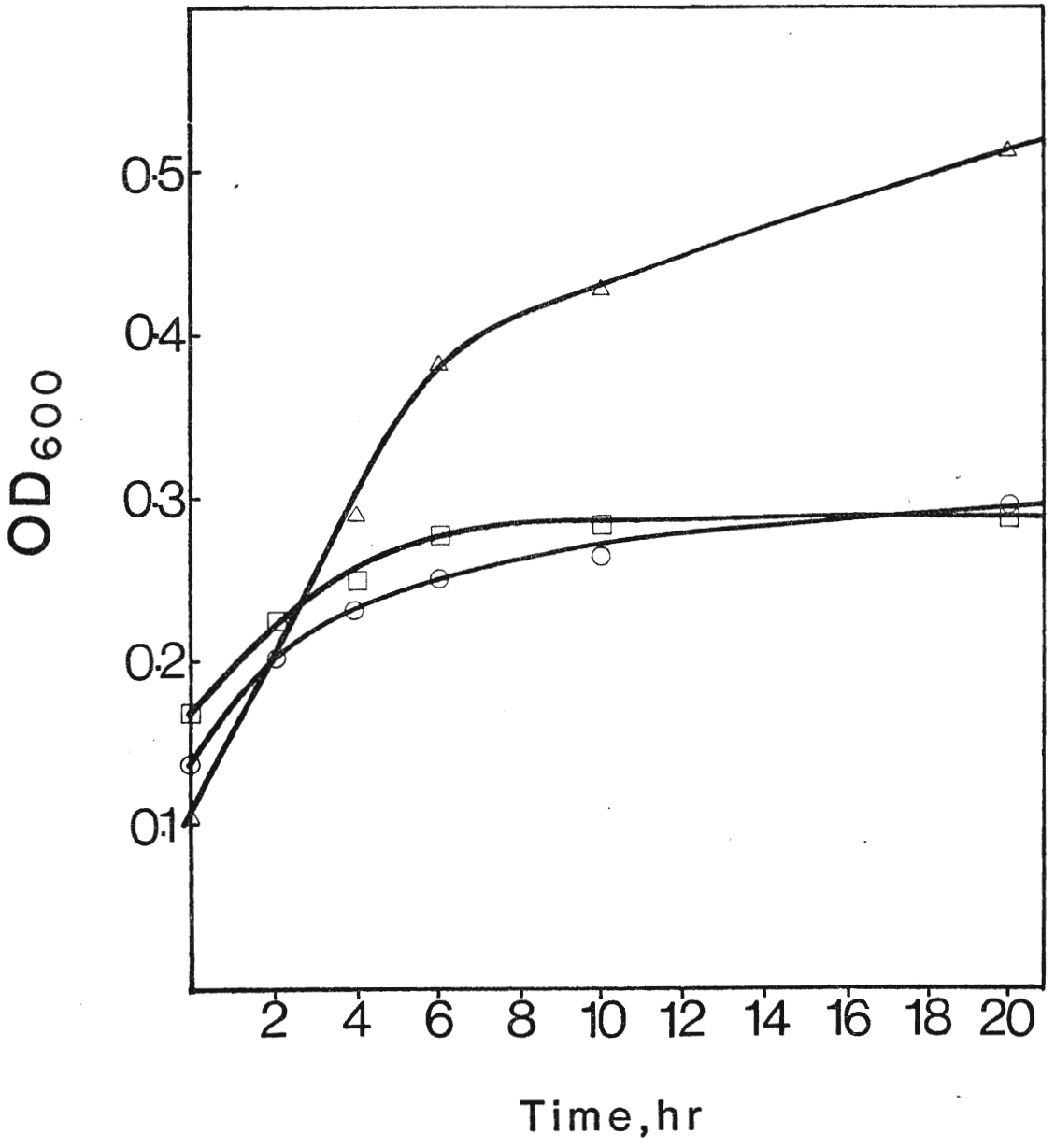
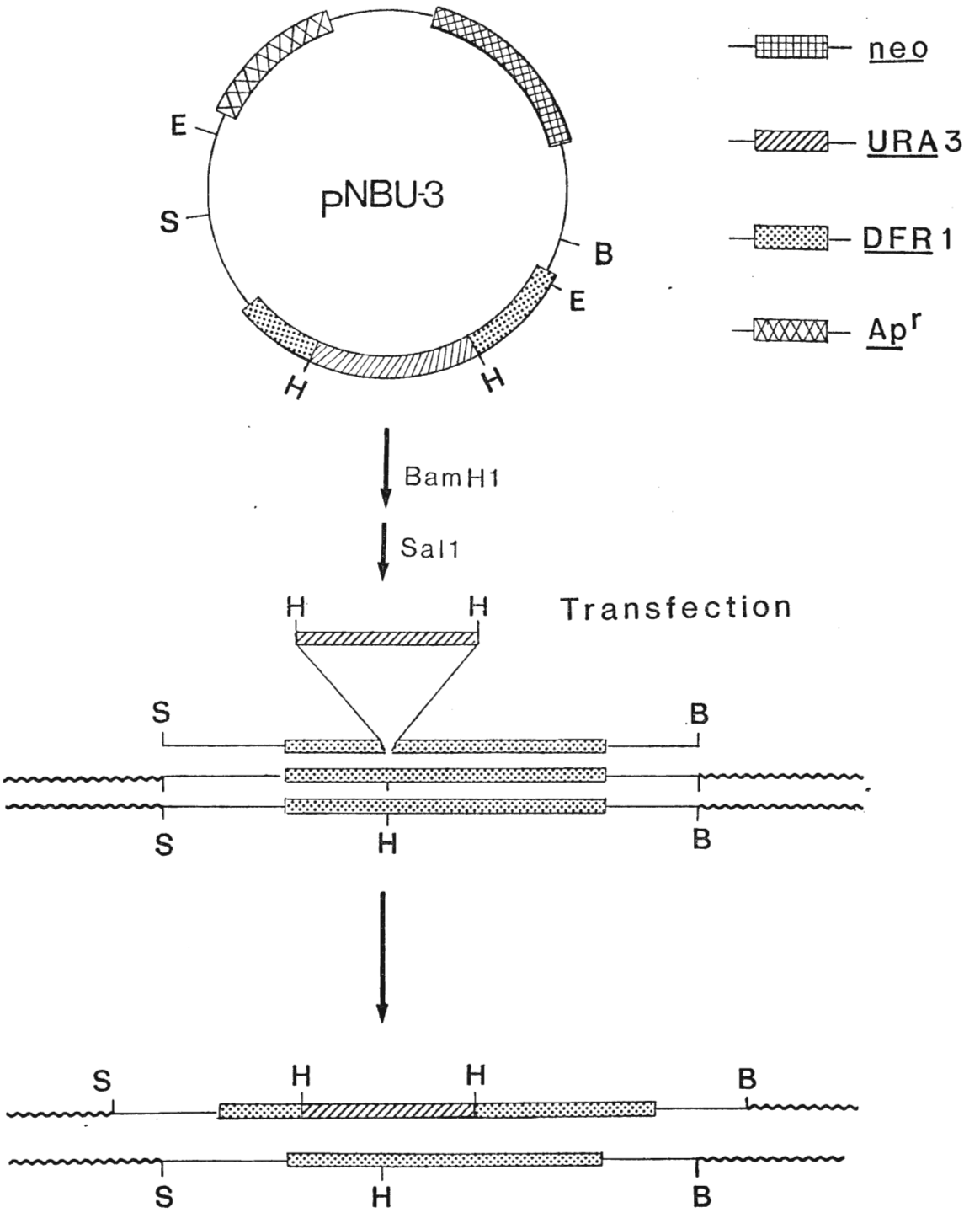

OD600
0·5
0·4
0·3
0·2
0·1
2
4
6
8
10
12
14
16
18
20
Time, hr

Figure 18 Disruption of DFR1 locus by plasmid pNBU-3.


pNBU-3
E
S
B
E
H
H
neo
URA3
DFR1
Ap^r
BamH1
Sal1
H
H
Transfection
S
B
S
H
B
S
H
H
B
S
H
B

attempted six times in a haploid strain M1/2B, no viable transfectants were recovered on SD medium supplemented with folinic acid, the assumed sole auxotrophic requirement of a yeast dhfr- strain.

This selection scheme was employed because it has been shown that the use of DHFR-specific inhibitor MTX combined with the antifolate sulfanilamide (SULF) can efficiently inhibit the growth of yeast cells, and that such an inhibitory effect could be overcome by supplementing the growth medium with folinic acid or the end products of one carbon metabolism (adenine, histidine, methionine and dTMP; see Fig. 19 and 20). Since wild-type yeast strains are impermeable to dTMP, a mutant strain permeable to dTMP is required for efficient "rescue" of cells grown in medium containing the antifolates. However, it has been demonstrated that efficient procedures used to isolate tup mutants have a strong selection for respiratory deficient petites which simultaneously failed to sporulate. Thus, such mutant strains were less convenient for the construction of a DHFR disruption. Due to these considerations, gene disruption experiment was first carried out in haploid strain M1/2B by using SD minimal medium supplied with folinic acid at 250 ug/ml, a standard level for other yeast folate-deficient mutant strains such as fol1, fol2 (Little and Haynse, 1979) and others (Barclay, personal communication). In six yeast transfection experiments in haploid strain M1/2B with plasmid pNBU-3, no matter how much of the DNA sample was used (from 10 to 70 ug for each transfection), and plasmid pNBU-3 was digested with only restriction enzyme

Figure 19. Growth rescue of MTX-treated yeast cells by folinic acid

Cells (M1/2B) were harvested from an exponential culture and inoculated at zero time into following media:

■ : minimal SD medium.
◆ : minimal SD medium containing sulfanilamide (5mg/ml) and methotrexate (20ug/ml).
▲ : minimal SD medium containing sulfanilamide (5mg/ml), methotrexate (20ug/ml) and folinic acid (250ug/ml).

Cell numbers were monitored by a Coulter counter.

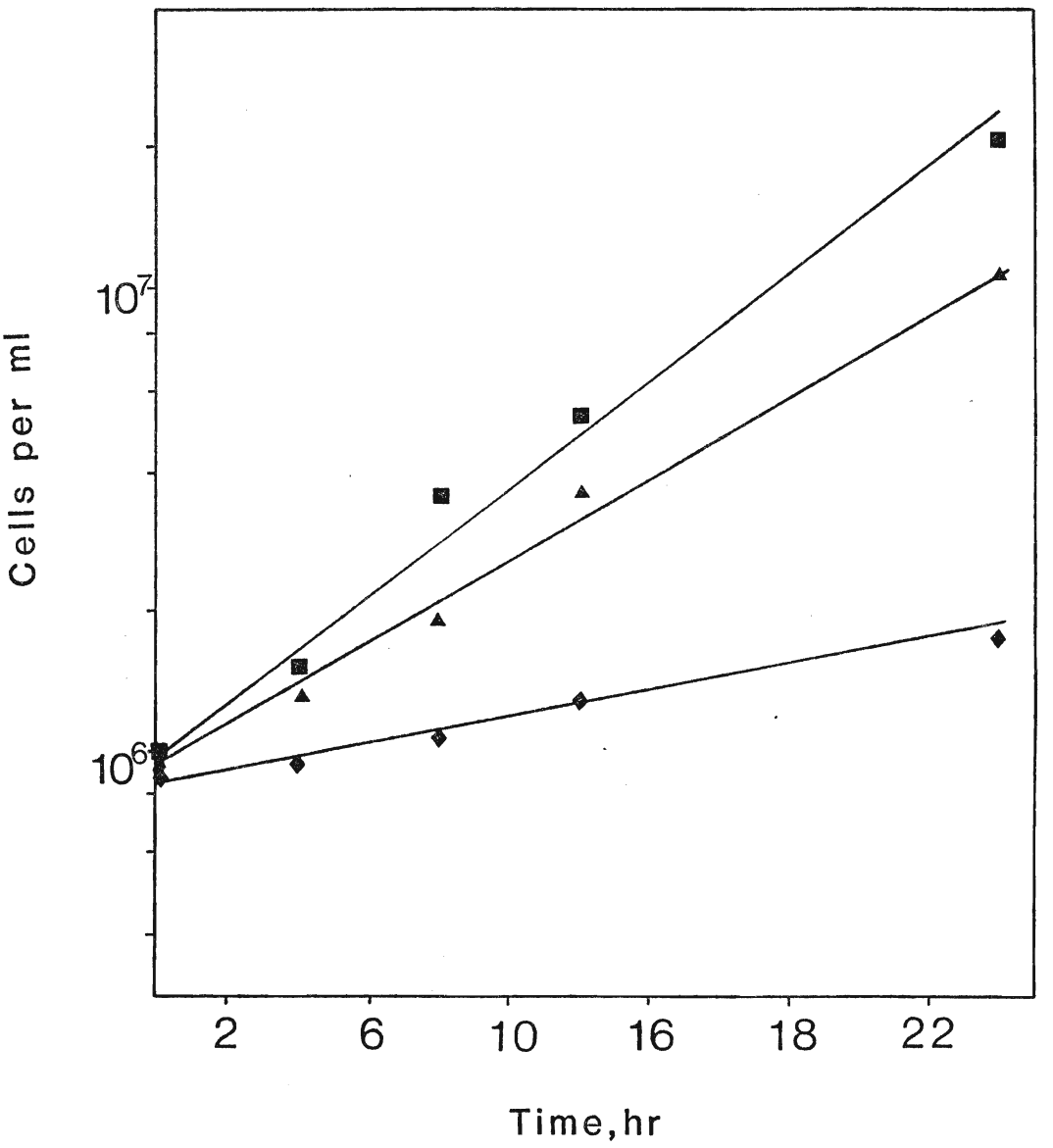
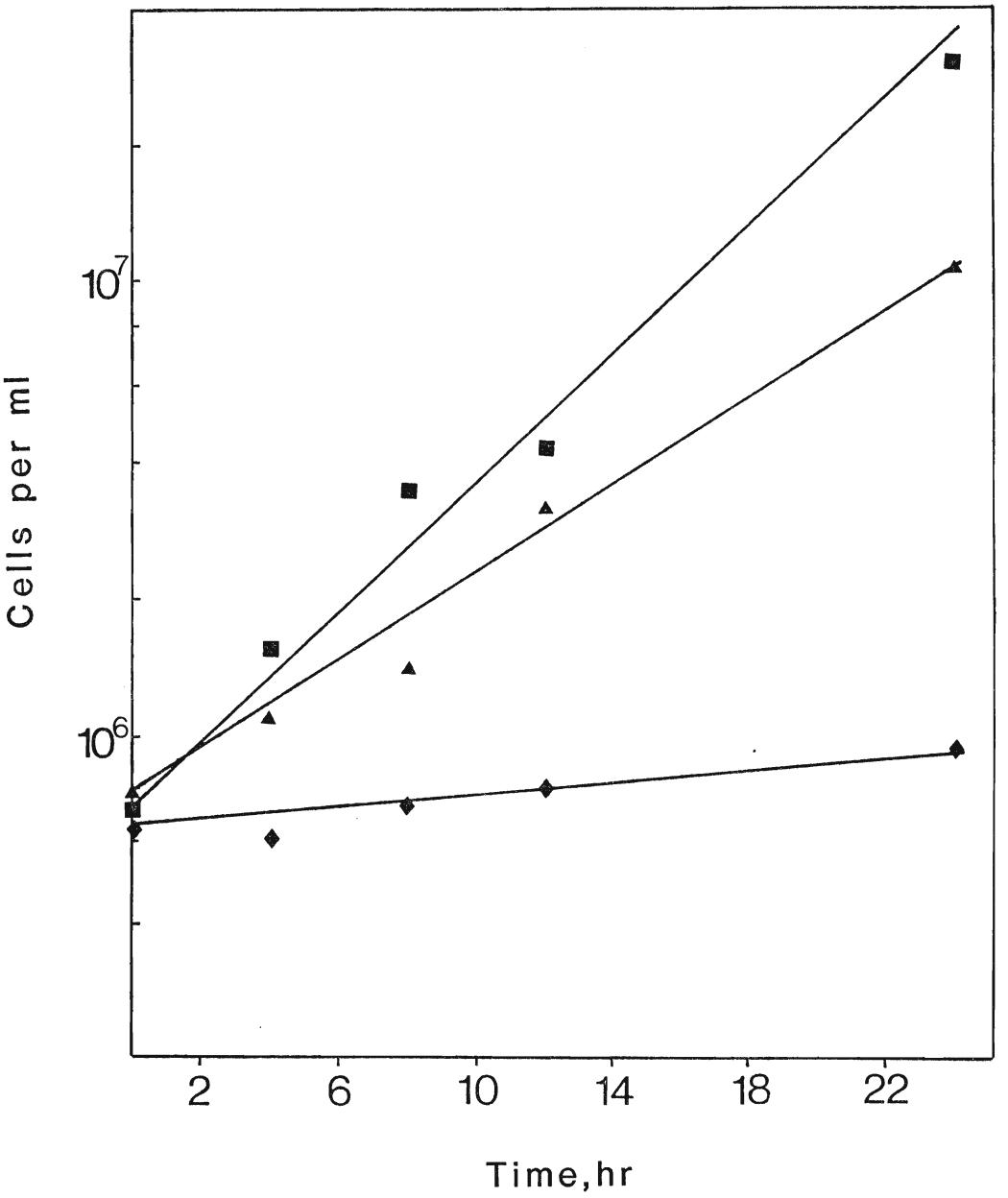

Cells per ml
10^7
10^6
2
6
10
16
18
22
Time, hr

Figure 20. Growth rescue of MTX-treated yeast cells by C1 metabolites

Strain TM1/2B permeable to dTMP was used. The cells were grown in following media:

■ : minimal SD medium.
◆ : minimal SD medium containing SULF (5mg/ml) and MTX (20ug/ml).
▲ : minamall SD medium containing SULF (5mg/ml), MTX (20ug/ml), adenine (30ug/ml), hisdine (20ug/ml), methionone (20ug/ml) and dTMP (25ug/ml).

The cultures were incubated at 34'C.


Cells per ml
10^7
10^6
2
6
10
14
18
22
Time,hr

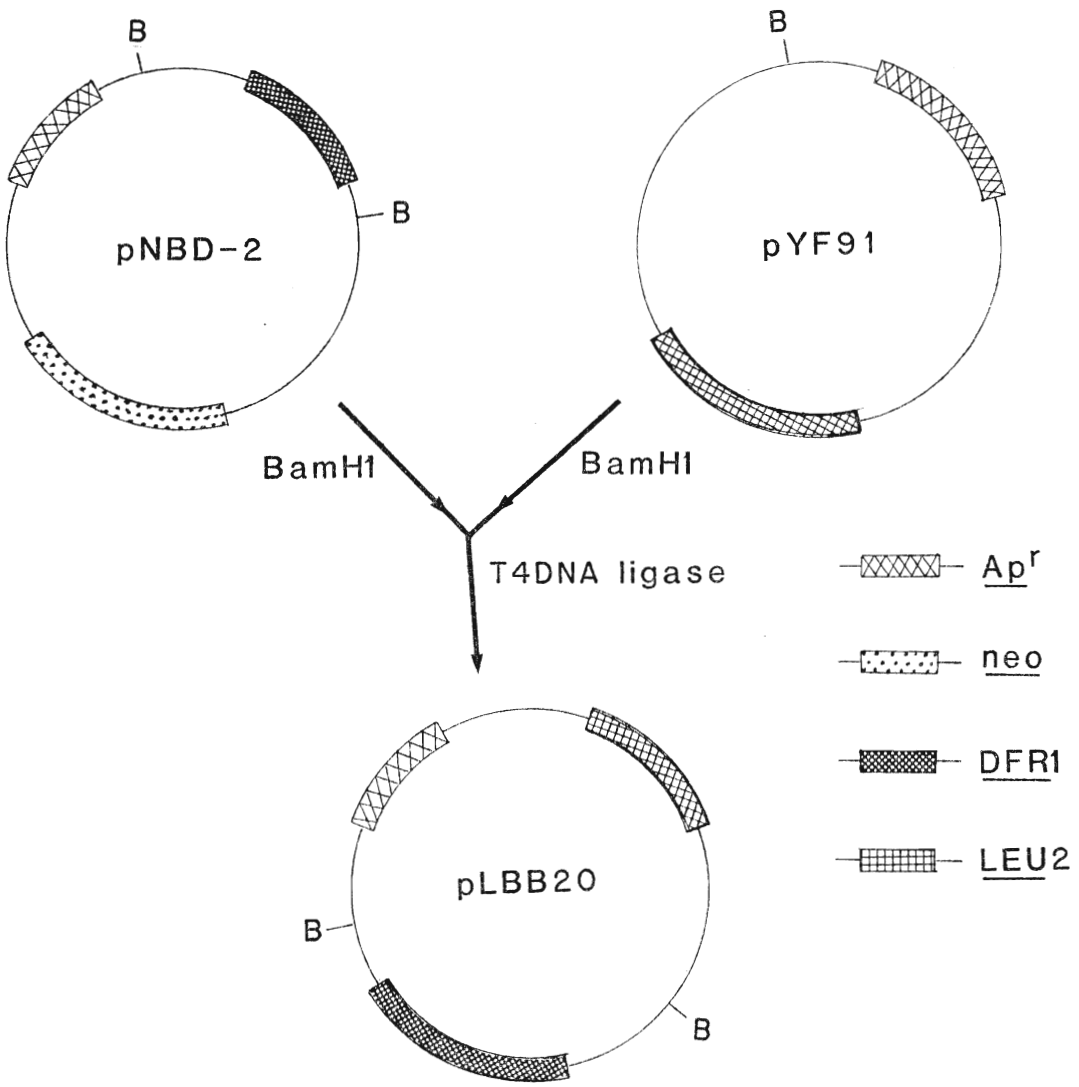
BamH1 or both BamH1 and Sal1 enzymes, or the Sal1/BamH1 restriction fragment containing the disrupted DFR1 gene by the URA3 gene was purified or not, no URA3 transfectants were obtained on the folinic acid-SD selection medium. In contrast, when the same plasmid was used to transform yeast cells without previous digestion with restriction enzymes and under the same selection condition, numerous uracil protrophs were recovered. These transfectants all carried a functional DFR1 gene in addition to the disrupted dfr1 DNA fragment due to a single crossover between the homologous sequences. These observations suggested that disruption of the DFR1 locus in a haploid yeast cell might result in the alteration of the host cell to a phenotype more complex than anticipated, ie. which failed to metabolize 5-CHO-THF or was inviable.

In order to confirm this interpretation, a diploid strain (TH4) was constructed by a cross of strain LP2729/4B with another haploid DS1/38. Thus, the diploid contained a homoallelic ura3 mutation suitable for the gene disruption experiment using plasmid pNBU-3 or pDEH-5. By using this diploid strain TH4 in two transfection experiments, URA3+ transfectants presumably heterozygous for the dfr1 disruption loci were easily isolated. Random spore analysis of diploid transfectants TH4/SBU-12 was performed on the medium same as used in the haploid transfection experiments and resulted in Ura-:Ura+ segregants in a ratio of 154:7. This strong bias for uracil auxotrophes in the meiotic products suggested that either the yeast DHFR had an essential function in yeast cell or alternatively that the Ura+ spore

clones harboring the dfr1 allele lacked the enzymatic activity necessary for the metablism of 5'-formyl THF (folinic acid).

In order to distinguish between these possibilities, a mutant strain of DBY747 permeable for dTMP was then isolated which would facilitate the use of another selective procedure with the end products of C1 metabolism. Additionally, plasmid pLBB20 was constructed (Fig. 21), which carried the functional DFR1 gene as well as a yeast LEU2 gene allowing selection of the plasmid. Thus, a haploid cotransfectant with plasmids pLBB20 and either plasmid pYEH-5 or plasmid pNBU-3 digested with BamH1 and Sal1 restriction enzymes should be viable due to the protection of the DFR1 carried by plasmid pLBB20. Subsequently, by curing the plasmid pLBB20 and following a selection on various conditions, it should be possible to characterize the phenotype of a dfr1 null mutant strain.

Figure 21. Construction of plasmid pLBB20.


B
B
pNBD-2
B
pYF91
BamH1
BamH1
T4DNA ligase
Ap^r
neo
DFR1
LEU2
pLBB20
B
B

DISCUSSION AND CONCLUSIONS

The nucleotide sequence of an 1.7 kb yeast genomic Sal1/BamH1 restriction fragment containing the DFR1 gene was determined by the Sanger dideoxynucleotide chain termination method. Previously, DNA sequences of some segments of this Sal1/BamH1 fragment were obtained in our laboratory (Nagel, 1985). Based on the analysis of sequence data from the EcoR1/BamH1 restriction fragment, a translation termination codon TGA, 27 bp downstream from the EcoR1 restriction site was identified. When this 27 bp region was translated into the possible amino acid sequences, in all three possible reading frames, a phenylanine moiety was observed to correspond to a consensus residue present in the carboxy-terminal region of several other DHFR proteins. In addition, some canonical sequences previously determined in other studies as having some function in transcription termination in yeast were identified downstream from the putative stop codon. Thus, this investigator suggested that the 3' terminus of the yeast DFR1 gene was located in this sequence.

The sequence data obtained in this study is in agreement with this assignment of the 3' terminus of the gene. In this study, complete sequence of the cloned Sal1/BamH1 genomic yeast DNA was determined which comprised 1784 bp. Within this restriction fragment, a single open reading frame of 633 bp was identified. This putative coding region of the yeast DHFR gene is sufficient to code for a polypeptide of 211 amino acids with a predicted molecular weight of 24,229.8 Daltons. Comparative

analysis of the predicted amino acid sequence with various DHFR proteins indicates that yeast DHFR exhibites significant homology with both prokaryotic and eukaryotic DHFRs. Many of the consensus residues are those implicated in the interaction of the protein with cofactor and/or various inhibitors.

In addition to providing details about the physical properties of the DFR1 gene product, the results of this study have also provided valuable information about the organization of the gene and the surrounding DNA sequences in the yeast genome. As described in the "Results" section, potential regulatory elements were identified in both 5' and 3' flanking regions of the gene. Two repeats of the general amino acid control UAS core sequence "TGACTC" were identified in the upstream region. If supported by further experimentation, this would suggest a novel regulatory mode for the DHFR protein in the general amino acid control system of yeast, and the first inclusion in this family of genes of an enzyme not directly involved in the intermediary metabolism of these amino acids. The DFR1:lacZ fusions constructed during this study will provide convenient molecular tools for the study in this regard. In addition to this putative regulatory sequence, analysis of the DNA sequence determined in this study suggests that the yeast DHFR gene might contain multiple control regions, reflecting alternate transcription initiation and termination sites. If supported by further experimental evidence, this would suggest that the yeast gene is regulated in a similar manner to DHFR genes of higher eukaryotes. In addition, homology of the 3' sequences of the DFR1 with

various proposed consensus sequences as important for RNA transcriptional termination and polyadenylation suggests that this gene would be a good experiment modeL to further investigate the function of these different consensus sequences in the regulation of yeast gene expression.

Analysis of codon usage of the yeast DFR1 gene was used to calculate the value of the Codon Bias Index (CBI). A CBI of 0.0083, implies that the gene is expressed at a relatively low level under normal physiological conditions. This result is in agreement with biochemical studies in which purification of the protein proved to be difficult (Wu, et al., 1976), and in which yields of the purified protein were extremely low. Presumably, this was due to the fact that the DHFR protein is present in very low quantities in yeast. Additional support for this view was obtained here in experiments utilizing the DFR1:lacZ fusions constructed during the course of this study. Thus, it was shown that the fusion peptides encoded on multicopy plasmids were expressed in yeast transfectants at low levels. Analysis of the codon usage of several known yeast regulatory genes, which all possess a CBI value very close to zero, has led the suggestion that, in eukaryotic as in prokaryotic organisms, the use of rare codons for specific amino acids serves as an attenuation mechanism in the expression of regulatory genes (Kammerer et al., 1984). The CBI value of 0.0083 for the DFR1 gene combined with the observation in the gene fusion studies mentioned above may imply that the yeast DHFR protein has, in addition to its catalytic activity, some regulatory function.

The sequence information of the DFR1 DNA fragment was used in construction of the two kinds of lacZ gene fusions. Both of them showed similar expression pattern in yeast, but obviously different in E.coli. The difference could be accounted for by the hetergeneous intracellular environment. The low level expression, in both bacterial and yeast cells, of the functional DFR1:lacZ fusions was indicated by a comparison with levels of some other yeast gene fusions with the lacZ, such as the CYC1:lacZ fusion carried by the plasmid pLG669-Z. Preliminary studies with the pHFZ-12 fusion plasmid demonstrated that UV light induced a transient increase in the expression of the gene. As pointed out previously, this increased seems to be not associated with gene amplification. Presently, it is unclear about the role of UV-induced expression of the yeast DHFR. Nevertheless, the finding demonstrated in this study may imply a noval function of DHFR protain in cellular responses to DNA damage.

Another novel aspect concerning the yeast DHFR function was revealed during the course of the gene disruption studies. A number of experiments were conducted in an attempt to construct a DFR1 gene disruption in a haploid host without success. Based upon the belief that MTX-treated cells represent a phenocopy of such a mutant, it was assumed that inclusion of exogenous folinic acid in the growth medium would satisfy the auxotrophic requirements of a strain harboring a DFR1 null allele. This seems not to be the case as a diploid transfectant heterozygous for the gene disruption with the URA3 DNA segregated 2:0 for

the Ura- phenotype, when spore clones were germinated on the folinic acid medium.

This result may suggest that yeast cells containing a non-functional DHFR fail to metabolize 5-formyl-THF. Such a phenotype has been observed in a DHFR deficient Chinese hamster cell line (L. Chasin, personal communication). Although there is no obvious involvement of the DHFR protein in either uptake or metabolism of folinic acid, it is conceivable that DHFR like other enzymes involved in C1 metabolism might form a component of a complex with several catalytic activities. Thus, a mutation in a gene coding for a contiguous protein might affect the function of a neighboring polypeptide. An alternative explanation is that DHFR has some essential function in yeast and that lack of this activity is incompatible with viability. Further characterization of the DHFR protein and its genetic regulation will be required to distingush between these posibilities.

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Appendix I. Published amino acid sequences of DHFR

Source	Reference
Mouse L1210 lymphoma	Stone et al. 1979
Mouse musculus	Chang et al. 1978
Porcine liver	Smith et al. 1979
Bovine liver	Lai et al. 1979
Chichen liver	Kumar et al. 1980
S. faecium I	Freisheim et al. 1977
S. faecium II	Gleisner et al. 1974
L. casei	Freisheim 1978
E. coli MB1428	Bennett et al. 1978
E. coli RT500 I and II	Baccanari et al. 1981
E. coli R67	Stone and Smith 1979
N. gonorrhoeae	Baccanari et al. 1984

Appendix II. Published DNA sequences of DHFR gene

source	Reference
Human	Chen et al. 1984
Mouse	Crouse et al. 1982
Chinese hamster lung	Melera et al. 1984
E. coli K12	Smith and Calvo 1980
E. coli R388	Swift et al. 1981
E. coli R483	Simonsen et al. 1983
E. coli R751	Flensburg and Steen 1986
L. casei	Andrews et al. 1985
L. major	Beverley et al. 1986
T4 phage	Purohit and Mathews 1984
Tn 7	Fling and Richards 1983